Mechanisms of stimulus-response coupling in platelet-activating factor stimulated platelets

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MECHANISMS OF STIMULUS-RESPONSE COUPLING IN PLATELET-ACTIVATING FACTOR STIMULATED PLATELETS

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ABSTRACT

The temporal and dose-response relationships of platelet-activating factor (PAF)-induced changes in the concentrations of cytosolic Ca^{++} ($[Ca^{++}]_i$), Ins(1,4,5)P_3 and sn-1,2-diacylglycerol (DAG) were examined in platelets. These signal molecules increased rapidly and transiently, with peak Ins(1,4,5)P_3 preceding maximal $[Ca^{++}]_i$ elevation in high-dose PAF stimulated platelets. In low-dose PAF stimulated platelets $[Ca^{++}]_i$ was elevated and dense granule constituents released, without any increase in Ins(1,4,5)P_3, DAG or phosphorylation of protein kinase C (PKC) substrate, suggesting that mechanisms other than polyphosphoinositide bisphosphate hydrolysis may be utilized for platelet activation by low concentrations of an agonist. Exogenous activation of PKC inhibited the elevation of DAG, Ins(1,4,5)P_3 and $[Ca^{++}]_i$ and also thromboxane (Tx) B_2 generation in PAF-stimulated platelets. In contrast the non-selective kinase inhibitor, staurosporine enhanced elevations of Ins(1,4,5)P_3, DAG, TxB_2 and the duration of the Ca^{++} signal in high-dose PAF-stimulated platelets but not low-dose, suggesting that endogenously activated PKC operates as a powerful negative-feedback regulator of signal molecules.

In agreement with staurosporine the potent and selective inhibitors of PKC, namely Ro 31-7549 and Ro 31-8220 potentiated TxB_2 generation and increased the duration of Ca^{++} elevation to a much greater extent in high-dose than in low-dose PAF-stimulated platelets. The Ro compounds also inhibited dense granule release and aggregation in PAF-stimulated platelets, demonstrating a bifurcating role for PKC in platelet signal transduction.

The presence of proteins cross-reacting to antibodies against annexins I, II and V was demonstrated in human platelets. These proteins were intracellular and not release from the granules upon platelet stimulation.

Tyrosine phosphorylation of proteins in PAF-stimulated platelets was characterised using anti-phosphotyrosine antibodies, and their role in signal transduction was investigated using the protein tyrosine kinase inhibitor genistein. Upon PAF stimulation several temporal waves of protein phosphorylation were

observed, which were differentially inhibited by genistein. Genistein inhibited elevation of Ins(1,4,5)P_3 and $[Ca^{++}]_i$ in PAF-stimulated platelets, suggesting a role for endogenously activated tyrosine kinase(s) in the early stages of signal transduction. Moreover, genistein also inhibited PAF-induced TxB_2 generation, dense granule release and aggregation.

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Abbreviations

5-HT (serotonin)	5-hydroxytryptamine
AA	arachidonic acid
ADP	adenosine diphosphate
ATP	adenosine trisphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanine monophosphate
$[Ca^{++}]_i$	cytosolic free calcium
$[Ca^{++}]_e$	extracellular calcium
DAG	sn-1,2-diacylglycerol
DHG	1,2-dihexanyl-sn-diacylglycerol (sn-1,2-dihexanoylglycerol)
EDRF	endothelial derived relaxing factor
EGF	epidermal growth factor
EGTA	ethylene glycol-bis[ß-aminoethyl ether] N,N,N',N'-tetraacetic acid
Fura-2-am	fura-2-acetoxymethyl ester
Fluo-3-am	fluo-3-acetoxymethyl ester
G-proteins	guanine nucleotide-binding proteins
GTP	guanine trisphosphate
GP	glycoprotein
HBT	HEPES buffered tyrodes' solution
$Ins(1,4)P_2$	inositol (1,4)-bisphosphate
$Ins(1,4,5)P_3$	inositol (1,4,5)-trisphosphate
$Ins(1,3,4,5)P_4$	inositol (1,3,4,5)-tetrakisphosphate
MLCKase	myosin light chain kinase
MLC	myosin light chain
NaF	sodium fluoride
NO	nitric oxide
PA	phosphatidic acid
PAF	platelet-activating factor
PAP	peroxidase antiperoxidase-antibody
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDGF	platelet derived growth factor
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PI	phosphoinositide

PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PRP	platelet rich plasma
PS	phosphatidylserine
$PtdIns(4,5)P_2$	phosphatidylinositol (4,5)-bisphosphate
PtdIns(4)P	phosphatidylinositol (4)-monophosphate
PtdIns	phosphatidylinositol
PTKase	protein tyrosine kinase
PTPase	protein tyrosine phosphatase
RMCE	receptor mediated calcium entry
ROC	receptor operated channel
SDS	sodium dodecyl sulphate
SMOC	second messenger operated channel
SS	staurosporine
TB	tris buffer
TBT-20	tris buffer containing 0.05% Tween-20
TLC	thin layer chromatography
TPA	12-0-tetradecanyl-13-phorbolacetate
Tx	thromboxane

1. INTRODUCTION

1.1 Blood platelets

The terminally differentiated anucleate cells, known as blood platelets are formed from fragments of cytoplasm which bud off from multinucleate bone marrow megakaryocytes (Penington, 1981). Circulating platelets are uniformly discoid shaped cells with an equatorial diameter of 2-4 μm and a thickness of around 1 μm. Although they have no nucleus, they retain within their cytoplasm many of the subcellular features common to other cell types, such as mitochondria, a dense tubular membrane system, microtubules, microfilaments, storage granules and lysosomes. In addition platelets also contain ribosomes and mRNA from their parent cell, but in general it is thought that they have a limited capacity for protein synthesis.

The circulating platelet count is species dependent, but in man is about 2 x 10^8/ml with a normal turnover rate of platelets of about 3.5 x 10^7/ml/day and a life span in the blood of about 7-10 days (Harker and Finch, 1969). However, platelet size, shape, count, rate of production and life span can all be altered in disease states, by diet manipulation or drug administration.

The first evidence of the discovery of blood platelets is a drawing made in 1842 by Addison where platelets were represented in relation to a fibrin network (Addison, 1840). By 1882 with the advances made in microscopy it was established that platelets played a central role in haemostatic plug formation in response to vascular injury reviewed in (Robb-Smith, 1967). The primary role of platelets is to arrest bleeding from areas of damage in blood vessel walls but they also have the capacity to participate in numerous other physiological and pathological processes and exhibit a wide range of cellular reactions.

Due to the ready accessibility of platelets as a relatively homogeneous population of cells, and the vast range of functions they possess, platelets have been widely used in the study of transport, secretion, cell adhesion and signal transduction. The introductory chapter to this thesis has reviewed the relevant

literature published up to the initiation of this study, in order to "set the scene" for the work to be executed.

1.2 Platelet Functional Responses

Platelets circulate in the blood as relatively quiescent cells only displaying morphological, metabolic and functional changes once they have become activated. Endothelial cells normally constitute an antithrombotic and anti-coagulant surface lining the blood vessels, although these properties depend partially on the absence of pro-coagulant and pro-thrombotic moieties. The endothelium is thought to be involved in vascular haemostasis by release of soluble mediators or the expression of membrane-bound molecules in response to external stimuli. The soluble mediators include prostacyclin (PGI_2) (MacIntyre *et al.*1978) and endothelium derived relaxing factor (EDRF) (Palmer *et al.*1987). PGI_2 functions by activating adenylate cyclase and increasing cAMP levels and is the most potent prostaglandin to inhibit platelet aggregation (Whittle *et al.*1985). EDRF, now believed to be nitric oxide, is also released from endothelial cells and is a potent vasodilator which activates guanylate cyclase in platelets and as a consequence also inhibits their activation (Radomski *et al.*1987a). PGI_2 and EDRF are thought to act synergistically to block platelet aggregation with EDRF also inhibiting platelet adhesion (Radomski *et al.*1987b). Endothelial cells also produce ecto-enzymes which catabolize the platelet agonist ADP in the plasma, thereby reducing the amount of agonist and increasing the level of adenosine which antagonizes platelet aggregation (Pearson and Gordon, 1985).

Platelets are capable of a very rapid response to a wide range of agonists, which activate these cells by occupying specific cell surface receptors. Interaction of physiological stimuli with their plasma membrane receptors initiates morphological changes in platelets including loss of their discoid shape and the formation of pseudopodia which is synonymous with platelet shape change and occurs concurrently with platelet adhesion (attachment of platelets to surfaces) and in advance of platelet aggregation (attachment of platelets to each other).

Depending on the nature and concentration of the exogenous stimuli, the morphological changes may be accompanied by the release of dense and α-granule constituents by exocytosis and the generation and release of products of the cyclo-oxygenase pathway.

The primary function of platelets is in haemostasis, and the interaction of blood platelets with the subendothelium is fundamental to this process. Blood vessels can become damaged with the resulting exposure of their subendothelium for various reasons, for example by physical damage (including the site of a by pass or transplant operation), by the disruption or ulceration of an atherosclerotic plaque, by the aging process which leads to calcification and damage of the vessels or simply at branches of coronary arteries where there is rapid and turbulent blood flow leading to subtle endothelial damage. Whatever the cause of the vascular injury, the exposure of structures of the subendothelium, in particular fibrillar collagen, elastin and microfibrils is followed by platelet adhesion to these components (Zucker and Borrelli, 1961). Adhesion is influenced by a number of factors including platelet number and blood flow rate which mediates platelet contact with the vessel wall. A number of glycoproteins on the surface of platelets play an important role in adhesion. Glycoprotein Ib (GPIb) serves as a platelet binding site for von Willebrand factor, this cofactor is synthesised almost exclusively by endothelial cells and is adsorbed to collagen or other components of the subendothelium therefore mediating platelet adherence (George *et al.*1984). The importance of GPIb in platelet adherence is emphasized in the bleeding disorder known as Bernard-Soulier syndrome, where in the absence of GPIb, adhesion of platelets to the subendothelium does not occur (George *et al.*1984). In parallel, the importance of von Willebrand factor is highlighted in von Willebrand's disease, where either von Willebrand factor is deficient from the plasma or is structurally or functionally defective resulting in diminished platelet adhesion to the subendothelium (George *et al.*1984). Less is known about the function of GPIa on platelets, however, the finding that platelets which do not express GPIa also fail to respond to collagen suggests that GPIa is a receptor for

collagen and in addition it has been reported to be important in platelet spreading rather than the initial adhesion (Nieuwenhuis *et al.*1985). Activation of platelets also causes expression of a glycoprotein complex, namely GPIIb/IIIa, which functions as a key receptor for the adhesive protein cofactors of platelet aggregation, but which is also a receptor for von Willebrand factor, fibrinogen and fibronectin which helps strengthen the adhesion process (Nurden, 1987).

When platelets are transformed from discs into spiny spheres following activation, the circumferential band of microtubules composed of tubulin together with actin and associated myosin which are major proteins of the platelet cytoskeleton contract forcing the granules towards the centre of the cell. This centralisation of the electron dense amine storage granules (dense granules), α-granules and lysosomes is generally thought of as a preparatory stage for their extrusion, termed the 'release reaction' reviewed in (Holmsen, 1978; Holmsen *et al.*1969). In general, a lower concentration of an agonist is required to release the constituents of α-granules than dense granules (Fuster *et al.*1987), however, ADP and adrenaline can induce the release of dense granules with little concomitant release of the α-granules (Mills *et al.*1968) whereas thrombin and collagen induce release of both types of granule (Holmsen *et al.*1969). The time course for the release of the granules is also different with the dense granules being released in advance of the α-granules (Holmsen *et al.*1969). A different signal transduction pathway is thought to exist for secretion of lysosome constituents than for secretion of the contents of the other two types of granule, with a role for PKC being proposed, and higher agonist concentrations being required for extrusion (Kajikawa *et al.*1983). The contents of the α-granules include fibrinogen, fibronectin, platelet factor IV, thrombospondin and von Willebrand factor which will promote coagulation, but other factors notably the potent mitogen, platelet derived growth factor (PDGF) and transforming growth factor (TGF) which may have wider implications, for example in atherogenesis and inflamation. The contents of the dense granules include 5-HT, calcium and ADP, factors which will

promote growth of the haemostatic plug. Table 1 lists the major contents of the three types of secretory granule.

ADP and serotonin released from dense granules are aggregatory agents in the presence of Ca^{++} and fibrinogen (Born, 1962; Macmillan, 1966; Vargaftig *et al.*1981). Release of these constituents (in particular ADP) will therefore amplify the original agonist signal and cause a second wave of platelet activation (Born, 1962; Macmillan, 1966). This further recruitment of platelets and formation of aggregates induced by ADP release has been described as the "first pathway" of aggregation reviewed in Vargaftig *et al.* (1981). Binding of ADP to specific membrane receptors induces platelet shape change and exposure of fibrinogen binding sites, which have been identified as GPIIb/IIIa receptors (Peerschke, 1985). The GPIIb/IIIa complex is a major component of the platelet plasma membrane, with about 50,000 copies exposed on circulating platelets, although they only bind fibrinogen when the platelets become activated (Nurden, 1987). Cross linking of fibrinogen to GPIIb/IIIa receptors on adjacent platelets with the formation of bridges is the key to the platelet aggregation mechanism regardless of the agonist involved (Peerschke, 1985). The crucial role played by GPIIa/IIIb in platelet aggregation is demonstrated in the condition of Glanzmann's thrombasthenia, an inherited disorder characterised by a deficiency or altered function of GPIIb/IIIa complexes in the platelet membrane resulting in failure of platelets to aggregate in response to any agonist, coupled with an abnormality of the clot reaction (George *et al.*1984).

Following platelet activation, the Ca^{++} dependent enzyme phospholipase A_2 is stimulated to release arachidonic acid (AA) from membrane phospholipids including phosphatidylcholine and phosphatidylserine. AA is then metabolised by the cyclo-oxygenase pathway to produce the prostaglandins PGG_2, PGH_2 and the non-prostaglandin thromboxane (Tx) A_2 (Bakhle, 1983; Hamberg *et al.*1975). The two pathways of AA metabolism are shown in figure 1. TxA_2 is formed by the action of thromboxane synthetase on PGH_2 and is the most abundant eicosanoid produced by platelets (Bakhle, 1983; Hamberg *et al.*1975).

GRANULE CONSTITUENTS

Dense Granules

anions	cations
ATP	5-Hydroxytryptamine
ADP	Ca^{++}
GTP	
GDP	

alpha-granules

plasma proteins	platelet specific proteins
fibrinogen	platelet factor IV
fibronectin	beta-thromboglobulin
albumin	platelet-derived growth factor
factor V	basic protein
factor VIII	
thrombospondin	
von Willebrand factor	

Lysosomes

Acid hydrolases
cathepsin D,E

Peroxisomes

catalase

Table 1 Major contents of platelet storage granules

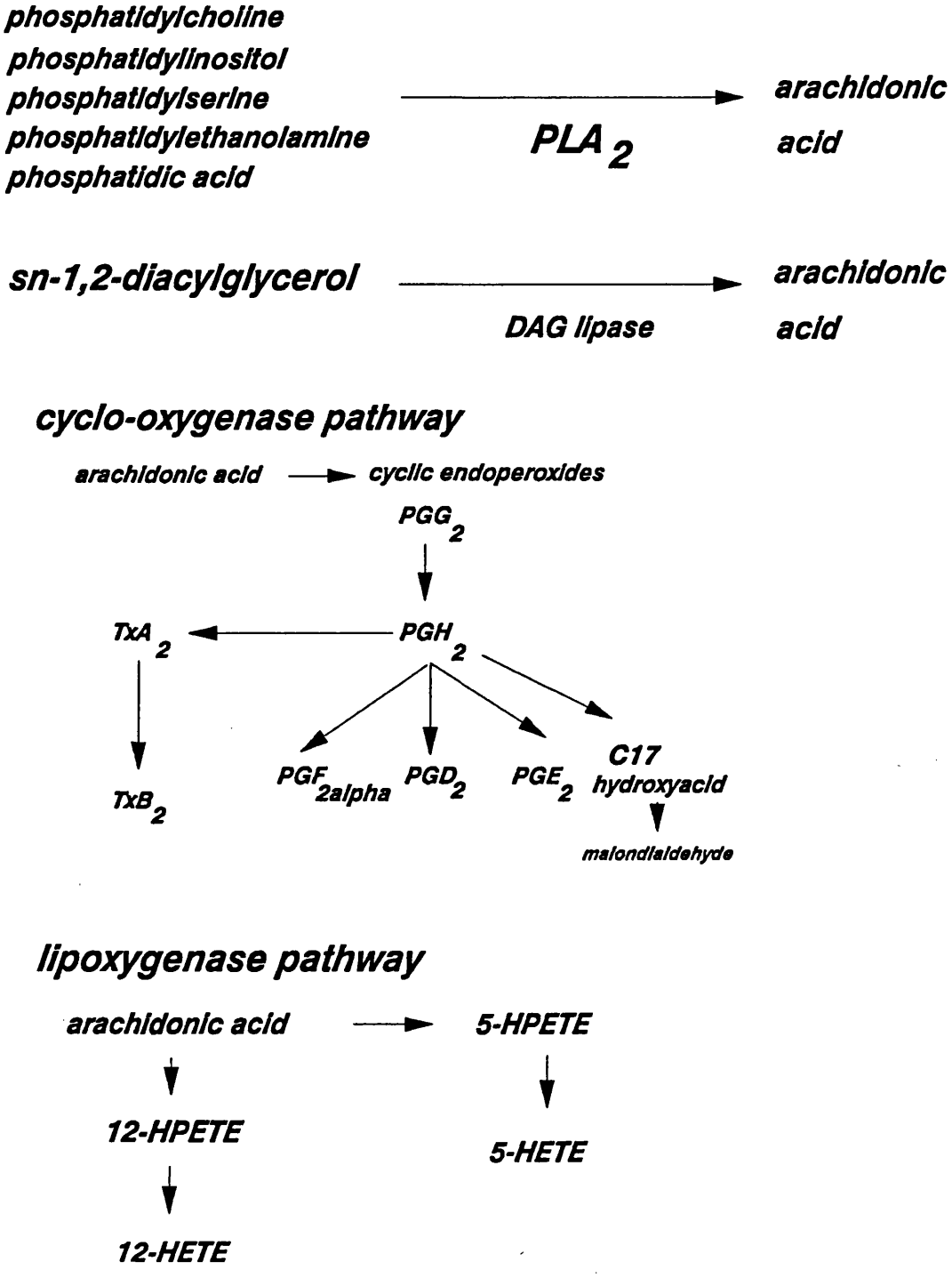


Figure 1 Mechanisms for the production and metabolism of arachidonic acid

TxA_2 is also a potent platelet agonist and vasoconstrictor, however it is unstable possessing a 1/2 life of only 30 seconds once it has been released into the blood, after which it is converted into non-biologically active TxB_2 (Hamberg *et al.*1975). The AA pathway has been described as the "second pathway" for platelet aggregation mediated by TxA_2 which acts as a positive feedback causing further recruitment of platelets to the haemostatic plug (Vargaftig *et al.*1981). Indeed an *in vivo* study in man has demonstrated that at the site of plug formation there is rapid and considerable platelet activation, with a 30 fold increase in TxA_2 over plasma values in the first minute after incision which can be inhibited by a low dose aspirin regimen (Kyrle *et al.*1986). The ratio of products from the cyclo-oxygenase pathway varies from one cell type to another, for example in contrast to platelets (which do not form PGI_2) endothelial cells form PGI_2 as their major cyclo-oxygenase product, whilst it is thought that they do not produce TxA_2, although TxA_2 production in cultured endothelial cells has been observed (Bakhle, 1983). Moreover, PGH_2 (which can also activate platelets by binding at the TxA_2 receptor (Morinelli *et al.*1987)) derived from platelets can also be synthesised into PGI_2 by endothelial cells causing an increase in platelet cAMP levels and therefore an inhibition of aggregation of neighbouring platelets (Marcus *et al.*1980). In addition to their inability to form PGI_2 platelets are also unable to form any of the leukotriene products of the lipoxygenase pathway of AA metabolism (Moncada and Higgs, 1986).

Activation of platelets and of other cell types involved in the haemostatic plug formation can induce the production and release of platelet activating factor (PAF) [1-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine] (Braquet *et al.*1987). PAF is one of the most potent platelet aggregating agents so far described with platelets possessing specific receptors for this agonist. Its production by platelets and other cells involved in haemostatic plug formation would suggest a high localised concentration (Zimmerman *et al.*1985; Touqui *et al.*1985). Experiments with inhibitors of prostaglandin synthesis and ADP scavengers have revealed a "third pathway" of platelet aggregation induced by thrombin independent of the first and

second pathways (ie ADP release and TxA_2 generation). Aggregation induced by PAF is not inhibited by either ADP scavengers or cyclo-oxygenase inhibitors suggesting an independent mechanism of platelet activation and therefore a possible additional pathway by which an original platelet response to a stimulus can be amplified (Vargaftig *et al.*1981). The release of PAF at the site of thrombus formation by a variety of activated cells, together with its potency as a platelet aggregating agent and its independence from dense granule release and eicosanoid generation for platelet aggregation has led to the suggestion that release of PAF may account for the "third pathway" of platelet aggregation (Vargaftig *et al.*1981). However, a number of studies have disputed PAF as the mediator of the "third pathway" of aggregation, demonstrating that inhibition of the cyclo-oxygenase pathway inhibits the second irreversible wave of PAF-aggregation, which is accompanied by dense granule release, and that desensitizing platelets to PAF by pretreatment with PAF has no effect on thrombin-induced aggregation induced by the so called "third pathway" of platelet aggregation (Kloprogge *et al.*1983).

During the process of platelet adhesion and aggregation the blood clotting mechanism may be initiated and thrombin generated, further promoting platelet aggregation and leading to the formation and polymerisation of fibrin. This will lead to stabilization of the platelet mass and prevent removal of the arterial thrombus by the high intravascular pressure and shear forces due to blood flow. The formation of thrombin is the end-point of a chain of enzymic events where one pro-enzyme is activated into an enzyme which in turn activates another proenzyme (Davie and Ratnoff, 1964). The proenzymes are present in the blood at very low concentrations but their activation is enhanced by their being adsorbed and concentrated on negatively charged phospholipid surfaces, with platelets *in vivo* being a major source of these surfaces (Mann *et al.*1987). Blood coagulation can therefore be considered as a series of surface catalysed events.

There are two pathways of coagulation, the intrinsic pathway where all necessary factors are present in the circulating blood and an extrinsic pathway

where a tissue component, specifically a lipoprotein called tissue factor or tissue thrombospondin initiates the activation of the coagulation cascade, leading to the formation of thrombin (Berrettini *et al.*1987). The two pathways merge at a later stage when factors VIII and IXa form a complex converting factor X into factor Xa for which platelet phospholipid (on the surface of aggregated platelets) and Ca^{++} are essential. A prothrombinase complex is formed when factor Xa is bound to a negatively charged phospholipid surface in the presence of factor V and Ca^{++}. Factor Xa associated with the prothrombinase complex converts prothrombin to thrombin, at a rate 300,000 fold greater than factor Xa alone, highlighting the important role for platelets in this process (Mann *et al.*1987). The principle actions of thrombin include the cleavage of fibrinogen (a constituent released from the platelet α-granules) to the fibrinopeptides and fibrin monomer which polymerizes to form an insoluble fibrin clot, and the activation of factor XIII to produce factor XIIIa which stabilizes fibrin. In addition, thrombin is one of the most potent physiological platelet aggregating agents and will therefore cause further recruitment of platelets to the aggregate and the release of the prothrombin factors V and VIII from the α-granules and the development of further prothrombin binding sites on the platelets (Bevers *et al.*1985).

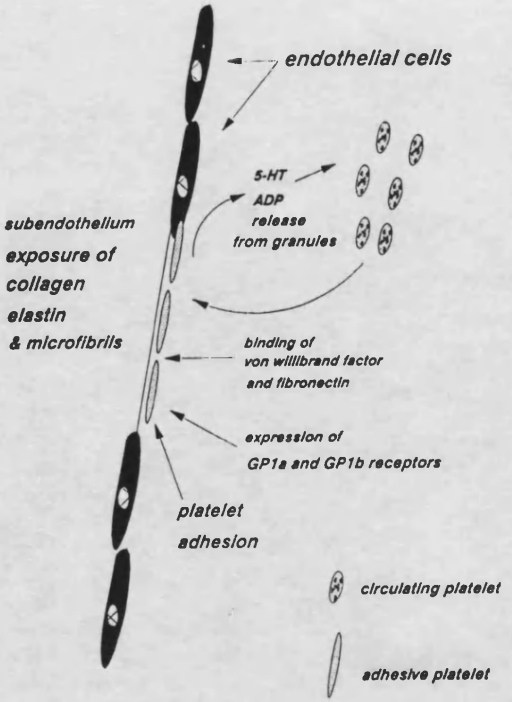
However, to counteract thrombus formation there are numerous endogenous inhibitors including the afore mentioned production of PGI_2 and EDRF from endothelial cells. Thrombus formation is also limited by the activation of protein C, fibrinolysis and the presence of antithrombin III (Ellis *et al.*1985). Thrombin in association with its endothelial cofactor thrombomodulin transforms protein C to activated protein C which limits thrombosis by destroying factor VIIIa and factor Va, a reaction which is accelerated by the presence of protein S (Ellis *et al.*1985). This activity initiates fibrinolysis by the release of tissue derived plasminogen activator which converts plasminogen to plasmin. Plasmin then cleaves fibrin into soluble fragments and degrades fibrinogen. Intravascular thrombosis is also limited by anti-thrombin III which is synthesised and expressed on the surface of endothelial cells and whose action is greatly increased in the presence of heparin,

which binds to the endothelial cell surface with a high affinity (Ellis *et al.*1985). Although heparin is not present in the blood, heparin like molecules (such as the glycosaminoglycan termed heparan) are associated with the vascular endothelium and potentiate the action of anti-thrombin III upto 20 fold (Marcum *et al.*1984). Endothelial cells bind free thrombin by means of a cofactor which favours a thrombin-antithrombin III complex. A deficiency in either protein C or anti-thrombin III is associated with thrombosis, providing evidence of the clinical relevance of this control mechanism (Ellis *et al.*1985). A schematic drawing of platelet adhesion, aggregation and blood clotting is given in figure 2.

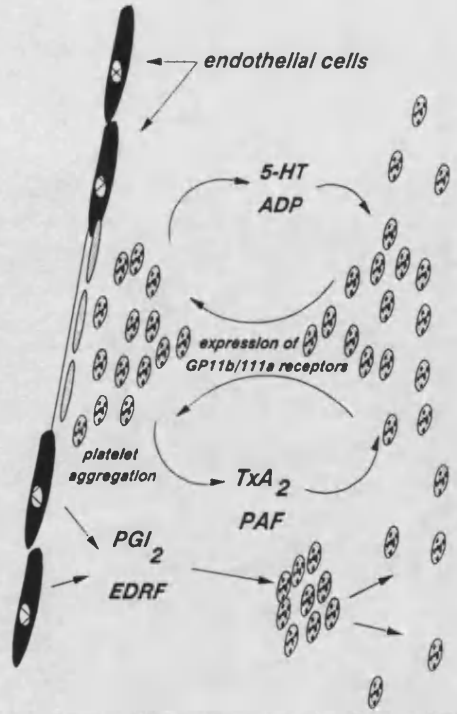
1.3 Platelets in Health and Disease

The most important physiological role of platelets in the circulation is in normal haemostatic processes. Platelets contribute to haemostasis in two ways, firstly they initiate the formation of a haemostatic plug at the site of vascular injury, and secondly they ensure efficient coagulation by release of factors from their storage granules which are involved in coagulation and also by providing a negatively charged phospholipid surface necessary for the activity of the prothrombinase complex (Mann *et al.*1987).

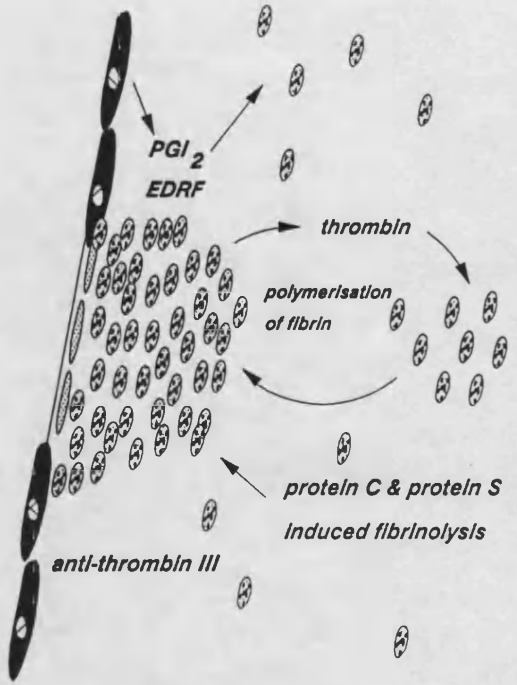
Over activity of the haemostatic process, together perhaps with an inability of the endothelial cells to maintain an antithrombotic barrier, are factors responsible for thrombus formation which is the pathological counterpart of haemostasis. The pathological consequences of thrombosis are serious and may result in occlusion of the blood vessel or parts of the thrombus may break off and occlude smaller vessels downstream from the original site. Thrombosis resulting in deprivation of oxygen to the tissues, particularly if the tissue in question is the brain or myocardium may lead to disability or death through stroke or myocardial infarction (Harker and Ritchie, 1980; Maseri *et al.*1980). Some medical conditions also appear to be predisposed to thrombus formation, for example renal vein thrombosis is a frequent complication of patients with nephrotic syndrome.



platelet adhesion at site of subendothelial exposure



Further platelet recruitment and aggregation



Thrombus formation and blood clotting

Fig 2 Schematic drawing of platelet adhesion, aggregation and blood clotting

Rejection of transplanted organs is also associated with occlusion of blood vessels due to platelet aggregants reviewed in Gordon and Milner (1976).

Another physiological function of platelets is their role in maintaining the integrity of the endothelial barrier by encouraging re-endothelialisation processes at injury sites and thereby decreasing vascular permeability (Gimbrone *et al.*1969). The detection of several growth factors released from the storage granules of activated platelets, including platelet derived growth factor (Ross, 1986), but in particular a novel platelet-derived factor capable of stimulating the proliferation of vascular endothelial cells, may have an important role in the re-endothelialisation process (Miyazono *et al.*1985).

Problems arise when the number of circulating platelets, normally maintained within a narrow range, either become to few (thrombocytopenia) or too many (thrombocytosis). The absence of platelets in thrombocytopenia, which can be a side effect of chemotherapy or radiation therapy, leads to haemorrhage and inability of blood to clot. This is exacerbated by the inability of the remaining platelets to maintain the integrity of the endothelial barrier, resulting in increased vascular permeability and further haemorrhage (Gimbrone *et al.*1969). The high density of platelets experienced in thrombocytosis is associated with thrombosis, indeed it is claimed that platelets may undergo spontaneous aggregation in the circulation under conditions of high density.

There is increasing evidence that platelets are also involved in the inflammatory process. Platelets have been demonstrated to undergo chemotaxis, phagocytose foreign particles and possess both IgE and IgG receptors, in addition they are an excellent source of a materials relevant to the inflammatory process which are both stored in their granules (eg platelet derived growth factor) or generated upon platelet stimulation (eg PAF). In addition, platelets accumulate at the site of vascular damage and release constituents which increase vascular permeability (Page, 1989). Indeed, in agreement with a role for platelets in the inflammatory response is the accumulating evidence that platelets participate in the pathological condition of asthma by either acting as inflammatory cells,

producing powerful spasmogens (including PAF) or interacting with other cells potentially involved in asthma (Morley *et al.*1984).

Release of the mitogen PDGF from the platelet granules is thought to play an important role in wound healing after tissue injury, however an abnormal release of this mitogen has been implicated in the genesis of pathological conditions, including the smooth muscle thickening which characterizes the occlusive disease of arteries, namely atherosclerosis (Ross, 1986). Atherosclerotic lesions are also sites of vascular damage, possibly as a result of plaque rupture, and therefore thrombus formation at these areas would be expected to contribute to the morbidity associated with advance atherosclerotic disease.

Most clinical studies investigating the effect of anti-platelet drugs have concentrated on their ability to prevent death in patients who are known to be at high risk from occlusive vascular disease, for example those already known to have myocardial infarction, or who are suffering from angina (The Aspirin Myocardial Infarction Study Research Group, 1980) and this may be where treatment is most necessary. However, many of these conditions are debilitating, and although anti-thrombotic drugs may increase the life of the patient, the underlying condition will not be cured, but simply controlled. It is possible therefore that other medium risk groups may also benefit from *preventative* treatment, for example in cases where a patient has a family history of premature coronary death, or where the patient has an illness such as diabetes or gout where they are predisposed to thrombotic disorders. Recently the finding that low dose aspirin resulted in a 44% reduction in the risk of myocardial infarction in a population of normal healthy males, clearly illustrates the benefits of an anti-thrombotic treatment, and further demonstrates the potential role for platelets in more advanced vascular disease (Steering Committee of the Physicians' Health Study Research Group, 1989). However, although the risk of death from haemorrhagic stroke was not statistically increased in that particular study, the fact that the numbers were higher in the aspirin treated group stresses that the beneficial effects of any treatment need to be tempered against the side-effects.

Indeed, the advantages of any antithrombotic treatment should be balanced carefully against its effects on normal haemostasis.

Occlusive vascular disease is the major cause of death in the Western world today, and there is substantial evidence that platelets are pivotal to the pathogenesis of stroke and myocardial infarction (Harker and Ritchie, 1980; Maseri *et al.*1980). Therefore the development of an antithrombotic treatment which is specific for platelets may reduce the huge number of fatalities which result from occlusive vascular disease every year.

1.4 Platelet agonists

Platelets possess specific receptors on their plasma membrane surface to enable them to respond to blood borne and surface ligands. Specific plasma membrane receptors for the excitatory agonists thrombin, TxA_2, PAF, collagen, ADP, vasopressin, serotonin (5-HT) and adrenaline have been detected on platelets using radioligand binding analysis, homologous desensitization and the use of specific antagonists. Platelets are non-electrically excitable cells (Hallam and Rink, 1985a), therefore in order for platelets to respond to an agonist there must be a mechanism of information transfer, whereby the events at the cell surface are able to influence the biochemical activity of the signal transduction pathway, resulting in platelet functional responses. The expression of these functional responses is thought to be controlled by the intracellular concentration of second messenger molecules including cyclic AMP, cytosolic free Ca^{++} ($[Ca^{++}]_i$) and sn-1,2-diacylglycerol (sn-1,2-DAG).

Agonists vary in their potency to elicit functional responses, the rank order of potency of platelet agonists found to induce $[Ca^{++}]_i$ elevation has been reported as thrombin > PAF = U44069 (TxA_2 mimetic) > vasopressin > ADP > 5-HT >> adrenaline = 0 and a similar rank order of potency has also been reported for induction of DAG elevation by these agonists (MacIntyre *et al.*1985). An important difference between platelet agonists is in their dependency on the positive feedback of cyclo-oxygenase products (in particular TxA_2) to induce

secondary aggregation and promote granule release. In the presence of inhibitors of the cyclo-oxygenase pathway, agonists such as ADP and adrenaline will only cause primary aggregation and no granule release, whereas more potent agonists such as thrombin and PAF can elicit irreversible secondary platelet aggregation and stimulate secretion of granules independently of cyclo-oxygenase product formation (Vargaftig *et al.*1981).

Platelets also possess receptors for inhibitory agonists including prostaglandins E_1, D_2 and PGI_2 which exert anti-aggregating actions in platelets by virtue of their ability to regulate adenylate cyclase activity and thus cAMP levels (Whittle *et al.*1985).

It is unlikely that platelets activated *in vivo* will be stimulated by just one agonist, but more likely that it will be the combined action of several different types of receptor occupation that will determine the degree of platelet activation. *In vitro* studies have demonstrated that platelet activation with more than one stimulatory agonist most often elicits a synergistic response (Adams, 1985), but obviously an *in vivo* situation would also include platelet-inhibitory agonists, particularly the release of PGI_2 from the endothelial cells.

The platelet agonist 1-alkyl-2(R)-acetyl-glycero-3-phosphorylcholine, termed platelet activating factor (PAF) is the platelet agonist used in most of the studies in this thesis. Evidence for the existence of specific receptors for PAF on platelets includes its extreme potency in triggering biological responses, homologous desensitization of the receptors and the finding that only the naturally occurring stereoisomer (R) is able to bind to the receptor (Prescott *et al.*1990). In addition, competitive binding studies utilising [^{3}H]PAF and unlabelled PAF or PAF antagonists, have confirmed both the presence and specificity of PAF receptors. These studies have identified a high affinity binding site for PAF on human platelets (K_D 37 nM; 240 - 1400 sites/cell) (Valone *et al.*1982; Kloprogge and Akkerman, 1984) and rabbit platelets (K_D 16-26 nM; 150 - 300 sites/cell) (Hwang *et al.*1986; Morrison and Shukla, 1989), although a low affinity binding site is also thought to exist (Valone *et al.*1982; Morrison and Shukla, 1989). Moreover,

binding of PAF to its receptor is integral to initiation of PAF-receptor coupled signal transduction (Morrison and Shukla, 1989), thought to be mediated via guanine nucleotide-binding proteins (G-proteins), and to PAF-induced functional responses (Kloprogge and Akkerman, 1984). Although termed *platelet*-activating factor, the agonistic effects of PAF are not limited to platelets, many other cell types including eosinophils, neutrophils and smooth muscle cells also possess specific receptors for PAF, and are activated upon PAF-receptor coupling (Braquet *et al.*1987).

Appropriate activation of numerous cell types including platelets (using thrombin, collagen or ionophore but not AA or ADP), neutrophils, leukocytes and endothelial cells (Braquet *et al.*1987; Touqui *et al.*1985; Zimmerman *et al.*1985) induces a rapid synthesis of PAF. Platelets co-operate with other cell types in the synthesis of PAF, for example, in response to appropriate stimuli both neutrophils and platelets can release PAF, however, the inclusion of platelets in a suspension of neutrophils results in a synergistic generation of PAF, due to the release of lyso-PAF from platelets which is converted to PAF by the neutrophils (Coeffier *et al.*1984). Although it is not feasible to measure the production of PAF *in vivo* at the site of haemostatic plug formation, it is likely that in this situation, where there is close interaction between different activated cell types most of which are capable of producing PAF, that the local concentration will be high enough to induce platelet activation, and indeed the extreme potency of PAF as a platelet aggregatory agent emphasises the need for only a very small amount of PAF to be released in order to produce significant biological effects *in vivo*. Moreover, the potential of endothelial cells to produce PAF, and the finding that PAF remains cell associated, may be significant in the interaction of the endothelium with circulating blood cells, in particular in platelet-endothelial cell interaction in response to vessel injury (Zimmerman *et al.*1985). Indeed, a recent study has demonstrated a role for PAF in primary haemostasis, reporting that the selective PAF-receptor antagonist L-652,731 prolongs the skin bleeding time in the rabbit,

suggesting that locally released PAF has a physiological role in the formation of the haemostatic plug (Livio *et al.*1988).

PAF is not stored in cells, but upon cell activation it is formed by a route known as the *remodelling* pathway (Prescott *et al.*1990). In this pathway, PAF is formed by the sequential activities of PLA_2 on alkyl-acyl-GPC (with the release of AA) to form lyso-PAF and the action of acetyltransferase on lyso-PAF to form PAF-acether, a pathway dependent on cell activation and the presence of Ca^{++}. Inactivation of PAF is via deacylation to lyso-PAF by acetylhydrolase and reacylation to alkyl-acyl-GPC by acyltransferase a pathway independent of the activation state of the cell (Figure 3). An alternative pathway for the production of PAF, is termed the *de novo* pathway. This pathway involves the synthesis of 1-O-alkyl-2-acetyl-sn-glycerol which is converted to PAF by a unique 1-alkyl-2-acetyl-sn-glycerol cholinephosphotransferase (Prescott *et al.*1990). This route of PAF production is absent from some cell types and its function has not yet been established, the finding that inhibition of PLA_2 suppresses PAF generation in both thrombin and ionophore stimulated platelets, suggests that the *remodelling* pathway is probably the main route of PAF formation in activated platelets (Benveniste *et al.*1982).

1.5 Signal transduction pathways

Since the development of a photometric method for the semi-quantitative measurement of platelet aggregation by Born in the 1960's all aspects of platelet research have evolved considerably (Born, 1962). At around this time it was also discovered that following stimulation, platelets released a number of constituents including ADP, serotonin and fibrinogen in a process termed "the platelet release reaction" reviewed in (Holmsen, 1978). The importance of ADP as an agent for platelet aggregation, and in particular the correlation between ADP released from platelets and secondary aggregation was also established (Macmillan, 1966).

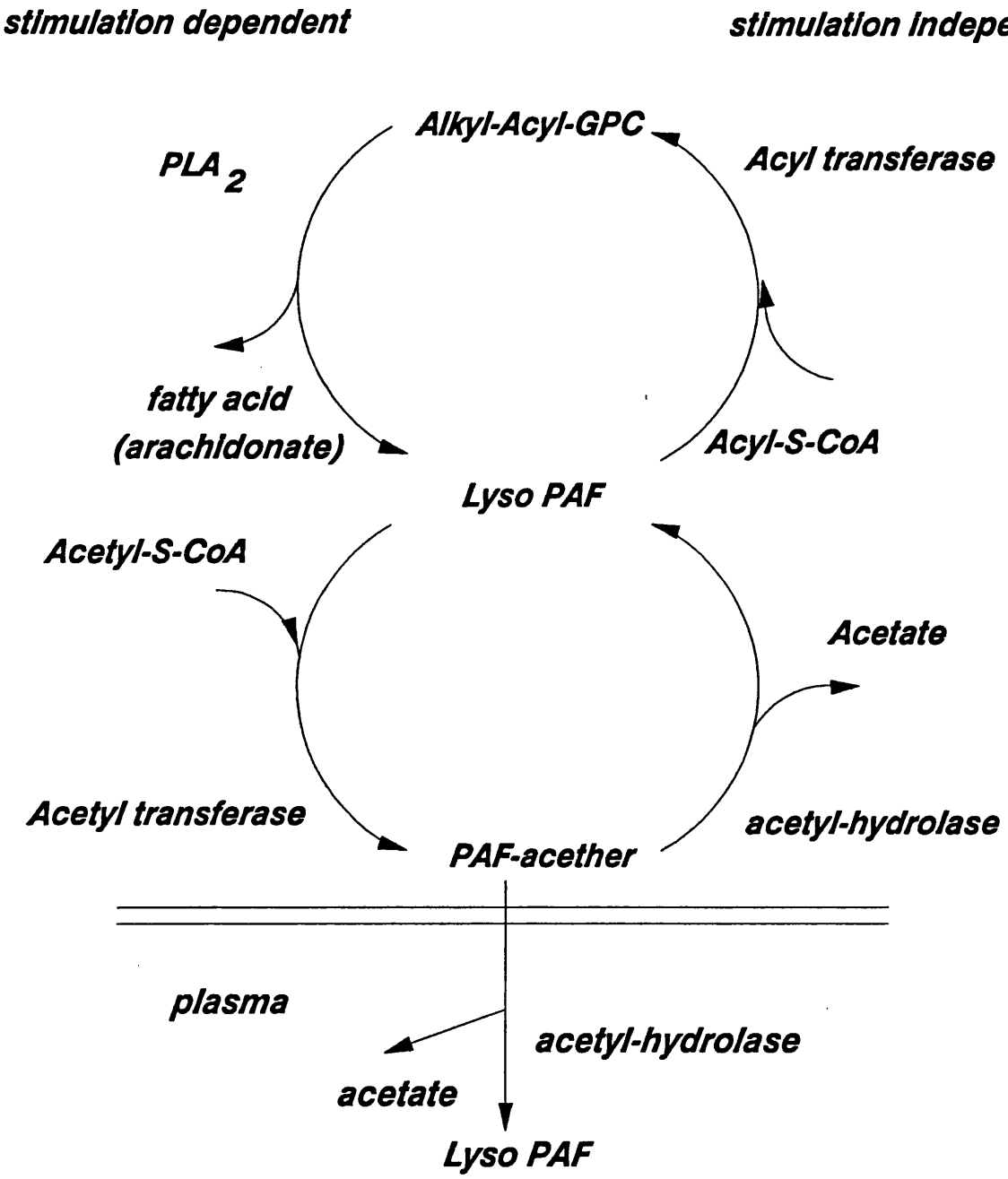


Figure 3 Metabolic cycle of PAF in platelets (Braquet etal 1987)

It was over a century ago that Ringer demonstrated the essential role for extracellular Ca^{++} in the normal functioning of the isolated frog heart which he believed functioned to stabilize the plasma membrane of the heart cell reviewed in (Rubin, 1985). Later, Ca^{++} was identified as a coupling factor between excitation and response in excitable tissues and it was realised that the role of Ca^{++} as a signal molecule was not confined to the heart, but was universal with activation of receptors linked to the polyphosphatidyl inositol (PI) cycle increasing ionized Ca^{++} in the cytosol and thereby inducing other responses including contraction of smooth muscle cells and exocytosis (Michell, 1975). In the 1970's the importance of Ca^{++} as an intracellular messenger in platelets was emerging due to the finding that increasing platelet $[Ca^{++}]_i$ with the use of divalent cation ionophores could elicit the same functional responses of platelet granule release and aggregation as a physiological agonist, suggesting that $[Ca^{++}]_i$ elevation could be responsible for mediating these functional events (White *et al.*1974; Feinman and Detwiler, 1974b).

Another important advance in that decade was the discovery of the cyclo-oxygenase pathway of AA metabolism, a mechanism leading to the production of prostaglandins and TxA_2 by platelets (Hamberg *et al.*1975), and the production of prostaglandins including PGI_2 by endothelial cells (Moncada *et al.*1976; MacIntyre *et al.*1978). TxA_2 was found to be both a potent platelet aggregating agent and a potent vasoconstrictor (Hamberg *et al.*1975), but in contrast PGI_2 inhibited platelet activity and possessed vasodilator properties (Moncada *et al.*1976). The release of ADP from the platelet granules together with the generation of TxA_2 by platelets were thought to be important mediators of platelet activation (Vargaftig *et al.*1981). Despite the advances made in platelet research in this decade, the nature of the intracellular biochemical signal transduction pathway responsible for triggering the basic platelet functional responses following agonist-receptor coupling was still a black box (Holmsen, 1978).

In an attempt to understand the mechanism of stimulus-response coupling intracellular events were examined, and it was found that activated platelets

selectively phosphorylated two proteins, one of 40-47 kDa and the other of 19-25 kDa, and that their phosphorylation was temporally related to the release of granule constituents (Haslam and Lynham, 1977). The lower molecular weight protein was identified as a substrate of the Ca^{++} and calmodulin-dependent myosin light chain kinase (Daniel *et al.*1984), whilst the higher molecular weight protein was identified as the major substrate of protein kinase C (PKC), an enzyme discovered and characterised by Nishizuka and colleagues and thought to play a pivotal role in platelet activation (Inoue *et al.*1977; Nishizuka, 1984). By the end of the decade it had been established that both intracellular Ca^{++} and protein phosphorylation were important mediators of platelet signal transduction (Lyons and Shaw, 1980).

At this time the pace of research into signal transduction quickened with attention being paid to the role of inositol phosphate turnover in platelets. The first evidence that phosphoinositide (PI) metabolism was involved in the response of cells to receptor-agonist coupling was produced by Hokin & Hokin in 1955 (Hokin and Hokin, 1955) where they demonstrated that acetylcholine accelerated the turnover rate of phosphoinositides in pancreatic tissue. It soon became clear that PI metabolism was coupled to stimulation of many different cell types, by many different agonists and that it was a mechanism closely related to $[Ca^{++}]_i$ elevation (Michell, 1979; Majerus *et al.*1985). In platelets it was soon established that this mechanism was commonly provoked by the coupling of agonists (except those which use cAMP as their second messenger) to their receptors (Lapetina, 1983; MacIntyre and Pollock, 1983). In 1979 it was discovered that hydrolysis of phosphatidylinositol (PtdIns) by phospholipase C (PLC) induced a transient accumulation of DAG in platelets (Rittenhouse-Simmons, 1979) and that the action of diglyceride lipase on DAG could release AA for metabolism by the cyclo-oxygenase pathway (Bell *et al.*1979).

The enzyme PKC was first described at this time, but had no obvious role in signal transduction (Inoue *et al.*1977). Later however, it was found to be a Ca^{++}

activated and phospholipid dependent enzyme, firmly linked to the signal transduction pathway by the discovery that DAG greatly increased the affinity of PKC for Ca^{++} thereby activating the enzyme (Nishizuka, 1984). Before the discovery of PKC, the potent platelet aggregatory action of the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), had been described (Zucker *et al.*1974). After the discovery of PKC the mode of action of phorbol esters was established when it was found that they could directly activate PKC, by substituting for DAG and greatly increasing the affinity of the enzyme for Ca^{++} (Castagna *et al.*1982). A pivotal role of PKC in signal transduction in numerous cell types was soon established (Nishizuka, 1984). The finding that phosphorylation of the major PKC substrate in platelets (40-47 kDa protein) correlated with release of various granule constituents, together with the finding that activation of PKC with phorbol ester alone also induced granule release suggested the central role of PKC in platelet signal transduction (Rink *et al.*1983; Kaibuchi *et al.*1983).

As well as producing DAG, hydrolysis of PtdIns(4,5)P_2 by phospholipase C in the PI cycle also produces Ins(1,4,5)P_3. It had been demonstrated earlier that activation of the PI cycle was closely associated with an increase in cytosolic Ca^{++} which was then able to evoke many physiological responses (Michell, 1975). The missing link in the signal transduction pathway was the association between activation of the PI cycle and an elevation of cytosolic Ca^{++}. In 1983 it was suggested by Berridge that Ins(1,4,5)P_3 which was rapidly formed upon activation of the insect salivary gland with 5-hydroxytryptamine may function as a second messenger in the control of the release of Ca^{++} from intracellular pools (Berridge, 1983). This study also demonstrated that it was the polyphosphoinositides (PPI) and not PI which were the primary targets of hydrolysis by phospholipase C. The fact that the hydrolysis of PtdIns(4,5)P_2 provided the only source of Ins(1,4,5)P_3 produced by the action of PLC, together with the finding that the hydrolysis of PtdIns(4,5)P_2 by PLC is Ca^{++} independent (and therefore elevation of Ins(1,4,5)P_3 can precede Ca^{++} elevation) (Billah and Lapetina, 1982) supported the theory of Berridge. A wealth of studies demonstrating that Ins(1,4,5)P_3 could release Ca^{++}

from the endoplasmic reticulum of numerous different types of permeabilized cell preparations, quickly substantiated the second messenger role of $Ins(1,4,5)P_3$ in regulating the mobilization of intracellular Ca^{++} (Berridge, 1984; Streb *et al.*1983; Berridge and Irvine, 1984). In particular, $Ins(1,4,5)P_3$ (at concentrations of 0.5 - 5 μM) was found to release $^{45}Ca^{++}$ from a Ca^{++}-transporting membrane vesicle fraction derived from human platelets (O'Rourke *et al.*1985), and from human platelets permeabilized with saponin (Brass and Joseph, 1985). Moreover, quantification of $Ins(1,4,5)P_3$ elevation in thrombin stimulated platelets indicated that adequate $Ins(1,4,5)P_3$ could be formed to induce the mobilization of Ca^{++} from intracellular stores (Rittenhouse and Sasson, 1985).

More recently, evidence has been produced for the involvement of guanine nucleotide-binding proteins in mediating the transduction of excitatory extracellular signals from receptors to the intracellular signal transduction pathway (Haslam and Davidson, 1984). Mechanisms of stimulus-response coupling are given in figure 4.

1.5.1 The role of Guanine nucleotide binding-proteins in signal transduction

Guanine nucleotide-binding proteins (G-proteins) mediate transduction of extracellular signals (which combine with receptors) to intracellular effector enzymes. Some of the first G-proteins to be identified were those associated with adenylate cyclase, namely G_s which stimulates adenylate cyclase and G_i which inhibits adenylate cyclase (Casey and Gilman, 1988). Platelets possess both G_s and G_i which are heterotrimeric proteins with G_s containing an α_s-subunit of 45 kDa and G_i containing an α_i-subunit of 41 kDa, but both possessing a $\beta\gamma$ dimer that is similar or identical in all G-proteins. Activation of G-proteins (by receptor occupancy) stimulates the replacement of GDP tightly bound to the α subunit by GTP, leading to dissociation of the protein into αGTP and $\beta\gamma$ components. Consensus sequences implicated in the binding and hydrolysis of GTP have been found in the α subunits. The GTP bound form is the active conformation which is then thought to stimulate its target enzyme. The intrinsic GTPase activity of αGTP terminates the interaction and the αGDP formed binds to $\beta\gamma$ again.

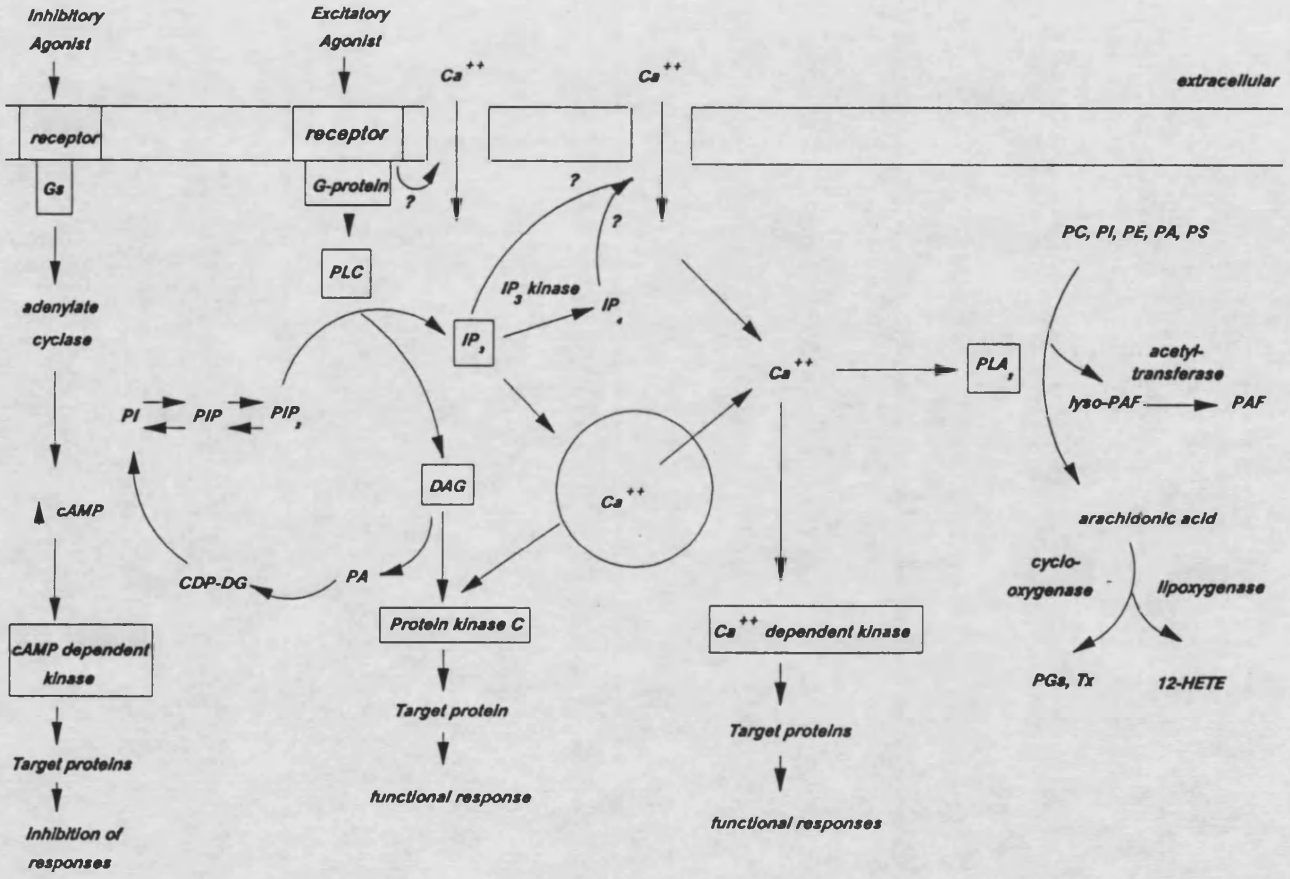


Fig 4 Schematic drawing of the major signal transduction pathways in platelet activation

This process continues as long as there is receptor occupancy thereby causing amplification of the original agonist-receptor binding signal, reviewed in Casey and Gilman (1988).

Evidence for the importance of closely related G-proteins in mediating the transmembrane signalling initiated by the occupancy of certain membrane receptors of stimulatory agonists is accumulating (Litosch and Fain, 1986). In particular there is evidence for GTP-binding proteins being directly involved in the regulation of PLC activity. This evidence includes the finding that GTP and its non-hydrolysable analogues such as GTPγS can stimulate PLC activity in different cell types including permeabilized platelets, where the stimulatory effects of thrombin are also enhanced (Haslam and Davidson, 1984; Hrbolich *et al.*1987). Conversely, GDPβS the non-hydrolysable analogue of GDP, causes a marked decrease in the activity of phospholipase C in permeabilized platelets (Kucera and Rittenhouse, 1988). Sodium fluoride (in particular the ALF^{4-} moiety) which activates G-proteins in intact cells has also been demonstrated to stimulate PLC activity in platelets (Kienast *et al.*1987).

1.5.2 Phospholipase C and the production of the two signal molecules Ins(1,4,5)P_3 and DAG

Activation of inositol phospholipid-specific phospholipase C plays a key role in signal transduction, and is a primary step in the major signal transduction pathway in platelets induced by a range of different agonists. Activation of PLC represents the link between agonist-receptor coupling and the PI cycle (Majerus *et al.*1985). It appears therefore that a wide range of agonists which bind to different receptors use this mechanism of signal transduction to induce the same basic platelet functional responses. The importance of PtdIns(4,5)P_2 hydrolysis is well established with evidence that the products, namely Ins(1,4,5)P_3 and DAG are second messengers responsible for mobilization of intracellular Ca^{++} and activation of PKC respectively (Berridge, 1984). However, pathways of platelet activation

independent of PI metabolism are also thought to exist for agonists such as ADP and adrenaline (MacIntyre *et al.*1985; Fisher *et al.*1985).

Early studies suggested that PtdIns was the main substrate of PLC, with its hydrolysis producing DAG and Ins(1)P (Rittenhouse-Simmons, 1979). However, it is now known that upon initial activation PLC causes preferential hydrolysis of PtdIns(4,5)P_2 producing both DAG and Ins(1,4,5)P_3 (Berridge, 1983; Majerus *et al.*1985). PtdIns and PtdIns(4)P are also substrates of PLC, but in addition the early hydrolysis of PtdIns(4,5)P_2 causes PtdIns to be sequentially phosphorylated by phosphatidyl-4-kinase to form PtdIns(4)P which is then phosphorylated by 4-monophosphate 5-kinase to replenish the pool of PtdIns(4,5)P_2. The PPIs are minor constituents of cellular lipid with PtdIns(4)P and PtdIns(4,5)P_2 respectively accounting for 1% and 0.3% of the total platelet phospholipid whilst PtdIns accounts for 5-7% (Siess, 1989). Hydrolysis of PtdIns(4,5)P_2 is Ca^{++} independent, whilst hydrolysis of PtdIns requires elevated intracellular Ca^{++} (Lapetina, 1983) suggesting different enzyme activities and indeed more than one PLC enzyme is now known to exist (Siess, 1989). Following platelet stimulation with an agonist such as thrombin a rapid loss of radioactivity in PtdIns(4,5)P_2 is observed, resulting in a subsequent elevation of Ins(1,4,5)P_3 and an increase in $[Ca^{++}]_i$. The increase in Ca^{++} will then allow the Ca^{++} dependent hydrolysis of PtdIns to proceed, the amount of PtdIns hydrolysed is ten fold greater than PtdIns(4,5)P_2 and may account for the majority of the DAG produced (Majerus *et al.*1985).

Both DAG and Ins(1,4,5)P_3 possess the characteristics of second messengers, they are formed rapidly, degraded rapidly and act at very low concentrations. In platelets agonist-stimulated elevation of DAG is rapid (Rittenhouse-Simmons, 1979; Preiss *et al.*1986) and transient due to both its phosphorylation by DAG kinase to phosphatidic acid (PA) (Tysnes *et al.*1988) and its degradation by DAG lipase to fatty acid (AA) and glycerol (Bell *et al.*1979; Mahaevappa and Holub, 1986). PA is converted into PtdIns through intermediate enzyme reactions (CTP-phosphatidate:cytidyl transferase and CDP-1,2-diacylglycerol-inositol phosphatidyl

transferase) as part of the PI cycle (Lapetina, 1983), whereas the AA formed, both by the action of DAG lipase and by the action of PA specific PLA_2 activity (Billah *et al.*1981), may be metabolised through the cyclo-oxygenase pathway in the generation of eicosanoids. Although the hydrolysis of (P)PIs is probably the major source of DAG it is possible that an additional source may be via the action of phospholipase D (PLD) on phosphatidylcholine (Loffelholz, 1989). In platelets however, the presence and role of PLD has yet to be verified (Rubin, 1988). The short-lived elevation of DAG is thought to activate PKC, a family of ubiquitously distributed iso-enzymes which have been implicated as having many different roles including the regulation of cell activation (Nishizuka, 1984).

The only well characterised source of Ins(1,4,5)P_3 is the hydrolysis of PtdIns(4,5)P_2 (Berridge, 1983). Ins(1,4,5)P_3 is rapidly elevated upon agonist-receptor coupling (Rittenhouse and Sasson, 1985; Berridge, 1983) and is transient due to its removal which is regulated by either phosphorylation to Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3 kinase or by degradation to Ins(1,4)P_2 by 5-phosphomonoesterase (Batty *et al.*1985; Connolly *et al.*1987; Mitchell *et al.*1989). Inositol phosphates are then further metabolised by specific kinases and phosphatases, with nearly 20 different inositol phosphates having been reported leading to the eventual formation of inositol (Majerus *et al.*1988). There is now considerable evidence that the transient elevation of Ins(1,4,5)P_3 is responsible for the mobilization of Ca^{++} from intracellular stores (Berridge, 1984; Streb *et al.*1983). In parallel to the formation of Ins(1,4,5)P_3 is the formation of much lower concentrations of inositol 1,2-cyclic 4,5-trisphosphate which is believed to be equipotent to Ins(1,4,5)P_3 at releasing Ca^{++} from intracellular stores (Tarver *et al.*1987).

Several factors are thought to be important in the regulation of inositol phospholipid hydrolysis by PLC, and these include the regulation of PLC activity by G-proteins, $[Ca^{++}]_i$ elevation and protein phosphorylation. A role for protein phosphorylation in regulating PLC activity has evolved for several different kinases, for example, exogenous activation of PKC decreases agonist induced PtdIns(4,5)P_2 hydrolysis and inhibits Ins(1,4,5)P_3 elevation, suggesting the PKC is exerting its

negative effect either at the level of PLC or an enzyme regulating the activity of PLC (Zavoico *et al.*1985). Indeed there are reports of phosphorylation of PLC isoenzymes by PKC both *in vitro* and *in vivo* reviewed in (Rhee *et al.*1989). Increased cAMP levels have also been shown to inhibit PI breakdown induced by agonists (Watson *et al.*1984). The mechanism by which cAMP-dependent kinase prevents PLC activation in stimulated platelets is not known, but may be through phosphorylation of a PLC isoenzyme (Kim *et al.*1989). Receptors for growth factors such as EGF and PDGF contain intrinsic protein tyrosine kinase (PTKase) activity (Hunter and Cooper, 1985) which is responsible for phosphorylation of PLC and in particular PLC*r*. Platelets are a rich source of PTKase(s) particularly $pp60^{c\text{-}src}$ (Golden *et al.*1986), therefore it is possible that PLC activity in platelets could also be regulated by tyrosine phosphorylation.

1.5.3 Cytosolic calcium as a second messenger

The resting intracellular free Ca^{++} concentration ($[Ca^{++}]_i$) is low in all animal cells with a concentration of between 10^{-7} and $3x10^{-7}$ M as opposed to an extracellular concentration ($[Ca^{++}]_e$) of Ca^{++} of around 10^{-3}M (Rink *et al.*1983). Small changes in the absolute amount of Ca^{++} in the cytosol therefore provide a large signal to background ratio. The 10,000 fold difference in Ca^{++} concentration between the cell interior and the extracellular milieu relies on strict homeostatic mechanisms to regulate the levels of $[Ca^{++}]_i$ (Rink, 1988).

In the unstimulated platelet it is thought that the resting membrane is very impermeable, although the mechanism by which the inevitable leak of Ca^{++} into the cell is counterbalanced by extrusion is still not known. One possibility for the removal of Ca^{++} would be the presence of Ca^{++}-ATPase activity which would allow Ca^{++} to be actively pumped from the platelet cytosol, however whereas this mechanism has been identified on the intracellular membranes in platelets it is not believed to be a feature of the surface membranes (Hack *et al.*1986). Another possibility for Ca^{++} homeostasis in resting platelets is the presence of Ca^{++}:Na^{+} exchange, complete replacement of Na^{+} with either K^{+} or choline was found to

cause a small increase in basal $[Ca^{++}]_i$ in resting platelets (Sage and Rink, 1986) supporting Ca^{++}:Na^{+} exchange across the surface plasma membrane of platelets (Rengasamy *et al.*1987). Moreover, a wide range of agonists induce a substantial elevation of $[Ca^{++}]_i$ which reaches a maximum concentration and then declines towards basal implying a powerful mechanism for the removal of Ca^{++} from the cytosol which may be either by resequestration into the intracellular stores (possibly by Ca^{++}-ATPase) or by extrusion across the plasma membrane against a concentration gradient. Activation of platelets with a physiological agonist such as thrombin, followed by the addition of phorbol ester which activates PKC induces a much more rapid return of $[Ca^{++}]_i$ to basal levels, suggesting a possible role for PKC in Ca^{++} homeostasis (Poll and Westwick, 1986a; Pollock *et al.*1987).

1.5.3.1 Advances in techniques for $[Ca^{++}]_i$ measurement

The earliest measurements of $[Ca^{++}]_i$ in living cells were made using the luminescent photoprotein aequorin injected into giant muscle cells. A decade later bis-azo absorbance dyes (including arsenazo III) were introduced together with selective microelectrodes as methods of monitoring $[Ca^{++}]_i$, these techniques were however, really only suitable for use in giant invertebrate cells reviewed in (Cobbold and Rink, 1987).

Knowledge of the role of $[Ca^{++}]_i$ in platelet signal transduction has developed in parallel with the techniques available for its detection. In the presence of $[Ca^{++}]_e$, divalent cation ionophores were found to induce platelet functional responses (Feinman and Detwiler, 1974b; White *et al.*1974) and moreover, platelet activation with the agonist thrombin was found to be associated with an increase in the uptake of $^{45}Ca^{++}$ (Massini and Luscher, 1976). The development of cell electropermeabilization, a technique which forms small pores in the plasma membrane without loss of protein constituents from the cytoplasm, but allows the $[Ca^{++}]_i$ to be clamped, demonstrated that in platelets an elevation of $[Ca^{++}]_i$ alone in the range 1 - 10 μM induced secretory exocytosis, but that in the presence of

thrombin the dose-response curve was shifted to the left (Knight and Scrutton, 1984). Development of the cell permeant fluorescent indicator quin-2 (Tsien *et al.*1982), allowed the dynamic changes in $[Ca^{++}]_i$ in response to an agonist to be measured for the first time in platelets (Rink *et al.*1982). Studies with quin-2 loaded platelets demonstrated that activation with a range of agonists including thrombin, PAF, vasopressin, ADP, TxA_2 (U44069) and PGH_2 (U46619) induces a transient elevation of $[Ca^{++}]_i$ which is larger in the presence of extracellular Ca^{++}, indicating both Ca^{++} influx across the plasma membrane and mobilization from intracellular stores (MacIntyre *et al.*1985; Hallam and Rink, 1985a).

Second generation fluorescent indicators include fura-2, fluo-3 and indo-1, which have improved properties over quin-2, are increasingly used and have confirmed the presence of two components of $[Ca^{++}]_i$ elevation in platelets. Indeed the use of dual wavelength fluorimetry using Mn^{2+} as a surrogate for Ca^{2+} (which causes a quenching of the dye fluorescence) has allowed Ca^{++} influx to be studied as a separate entity to mobilization (Alonso *et al.*1989). Aequorin, which was one of the earlier dyes discovered may still prove to be a useful tool as it is thought to measure localised changes in $[Ca^{++}]_i$ as opposed to the average rise in $[Ca^{++}]_i$ which is measured by fluorescent indicators such as fura-2. Indeed, in aequorin loaded platelets an increase in $[Ca^{++}]_i$ has been detected upon stimulation with collagen, adrenaline and PMA (Erne *et al.*1987) whereas no such increase is observed with fura-2 or quin-2 (Rink *et al.*1983; Watson *et al.*1985; MacIntyre *et al.*1985).

1.5.3.2 Mechanisms of $[Ca^{++}]_i$ elevation

The three main mechanisms thought to exist to elevate $[Ca^{++}]_i$ in animal cells are (i) $Ins(1,4,5)P_3$ induced Ca^{++} mobilization from intracellular stores analogous to the endoplasmic reticulum (termed the dense tubular system in platelets), (ii) receptor-mediated Ca^{++} entry across the plasma membrane and (iii) voltage-gated Ca^{++} entry across the plasma membrane in electrically excitable cells. Platelets are electrically non-excitable cells and as such do not possess voltage-dependent

Ca^{++} channels (Hallam and Rink, 1985a). However, there is evidence that in agonist stimulated platelets Ca^{++} is elevated by both mobilization (Brass and Joseph, 1985; Berridge, 1984) and influx across the plasma membrane (Hallam *et al.*1984; Hallam and Rink, 1985a).

1.5.3.3 Ins(1,4,5)P_3 induced $[Ca^{++}]_i$ mobilization

The close association between receptor activation, Ins(1,4,5)P_3 formation and the elevation of cytosolic Ca^{++}, led to the theory that Ins(1,4,5)P_3 was responsible for the mobilization of Ca^{++} from intracellular stores (Berridge, 1983). There is now sufficient evidence to suggest that this is correct (Berridge and Irvine, 1984; Berridge and Irvine, 1989) including the finding that exogenously added Ins(1,4,5)P_3 can stimulate the efflux of Ca^{++} from internal stores of many permeabilized cell preparations including platelets (Berridge, 1984; Streb *et al.*1983; Berridge and Irvine, 1984; Brass and Joseph, 1985; O'Rourke *et al.*1985). In addition, inositol 1,4,5-trisphosphorothioate, a non-hydrolysable synthetic analogue of Ins(1,4,5)P_3, has also been found to mobilize Ca^{++} from intracellular stores of several different permeabilized cell preparations including hepatocytes and is only three times less potent than Ins(1,4,5)P_3 (Taylor *et al.*1989). Moreover, in support of a second messenger role for Ins(1,4,5)P_3 is evidence of specific, saturable and reversible binding sites for ^{32}P-Ins(1,4,5)P_3 in permeabilized hepatocytes and neutrophils (Spat *et al.*1986) and more recently the solubilization, purification and characterisation of an Ins(1,4,5)P_3 receptor from rat cerebellum (Supattapone *et al.*1988).

1.5.3.4 Receptor Mediated Calcium Entry

The contribution of influx of extracellular Ca^{++} and of mobilization of intracellular Ca^{++} towards total elevated $[Ca^{++}]_i$ has been evaluated in several platelet studies by replacing extracellular Ca^{++} with the Ca^{++} chelator EGTA (MacIntyre *et al.*1985; Hallam *et al.*1984; Hallam and Rink, 1985a). In these studies elevation of $[Ca^{++}]_i$ induced by agonists including thrombin, PAF,

vasopressin, U44069, ADP and 5-HT was markedly impaired when extracellular Ca^{++} was replaced with EGTA implying that influx of Ca^{++} across the plasma membrane is a major component of agonist-induced $[Ca^{++}]_i$ elevation. Moreover, the elevation of $[Ca^{++}]_i$ is prolonged in the presence of extracellular Ca^{++}, suggesting that the Ca^{++} influx component is responsible for sustaining the increase in $[Ca^{++}]_i$ (Pollock and Rink, 1986). An influx component of $[Ca^{++}]_i$ elevation in platelets is also supported by the observation that extracellular $^{45}Ca^{++}$ is taken into platelets upon agonist stimulation (Massini and Luscher, 1976). Maximal stimulation of Ca^{++} influx in thrombin stimulated platelets occurs in the first few seconds, indicating rapid closure of the Ca^{++} channels (Poll and Westwick, 1986b).

In order to distinguish Ca^{++} entry from mobilization of Ca^{++} from intracellular stores the use of Mn^{2+} as a Ca^{++} surrogate in conjunction with dual wavelength excitation fluorimetry have been developed to use in conjunction with cells loaded with the fluorescent dye Fura-2 (Merritt and Hallam, 1988; Alonso *et al.*1989). At an excitation wave length of 340 nm, Mn^{2+} acting as a Ca^{2+} surrogate (at least through some pathways) will move into cells and quench the fluorescence, whilst Ca^{2+} will increase the fluorescence signal. At a wavelength of 360 nm however fluorescence of fura-2 is insensitive to $[Ca^{++}]_i$ and therefore the only signal after cell stimulation will be quenching caused by an influx of Mn^{2+}. Employing this method Merritt and Hallam demonstrated that stimulation of platelets with thrombin resulted in an increase in $[Ca^{++}]_i$ which was due to both release from internal stores and influx across the plasma membrane (Merritt and Hallam, 1988).

Little is known about the regulation of the Ca^{++} channels which exist to transport Ca^{++} across the plasma membrane and into the cytosol upon platelet stimulation. Two general types of receptor mediated Ca^{++} entry have been proposed, firstly entry which is modulated indirectly through some internal diffusible messengers (believed to be inositol phosphate(s)) and which is termed a second messenger operated channel (SMOC) and secondly entry which is

regulated directly by the receptor and termed a receptor operated channel (ROC) (Berridge and Irvine, 1989). Using dual wave length fluorimetry and Mn^{++} as a Ca^{++} surrogate, various agonists were found to cause a different rate and extent of Ca^{++} channel opening in platelets (Alonso *et al.*1989). ADP and PAF were found to induce rapid and transient opening of the Ca^{++} channels, whereas thrombin caused a rapid and maintained opening and collagen caused a delayed opening of the channels (Alonso *et al.*1989). Using this method it would be possible in some cases to differentiate between Ca^{++} influx regulated by a SMOC and influx which was through a ROC and which did not depend on the elevation of second messengers. Therefore, if Ca^{++} influx, detected by a quenching of fluorescence due to Mn^{++} influx, preceded intracellular Ca^{++} mobilization this would predict the presence of a ROC. Indeed ADP induced Mn^{2+} entry has been found to clearly precede internal Ca^{++} discharge suggesting a ROC may be responsible for at least part of the Ca^{++} influx component in ADP stimulated platelets (Sage *et al.*1989).

It has recently been demonstrated that Ins(1,4,5)P_3 alone can activate ion channels responsible for mediating Ca^{++} influx in mitogen stimulated T-cells (Kuno and Gardner, 1987). Moreover, studies on sea urchin egg cells have demonstrated a role for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_3 acting together as messengers in the regulation of Ca^{++} influx (Irvine and Moor, 1986), whilst a combination of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is required to evoke a sustained increase in Ca^{++}-activated K^+ current which is dependent on external Ca^{++} in mouse lacrimal acinar cell (Morris *et al.*1987). The role of second messengers as regulators of Ca^{++} entry in platelets is unknown, however recently Ins(1,4,5)P_3 has been linked to the opening of Ca^{++} channels in the platelet plasma membrane (Rengasamy and Feinberg, 1988).

1.5.3.5 Calcium dependent biochemical and functional responses

In the presence of extracellular Ca^{++}, divalent cation ionophores induce similar functional responses to physiological agonists in platelets proposing that Ca^{++} is a principle mediator of activation-response coupling (White *et al.*1974; Feinman and Detwiler, 1974a; Lapetina *et al.*1978). Studies employing ionophores have been used to determine the $[Ca^{++}]_i$ required to elicit a particular response. Shape change and myosin phosphorylation were found to be most sensitive to $[Ca^{++}]_i$ elevation, followed by aggregation and secretion, whilst the response requiring the highest $[Ca^{++}]_i$ was PLA_2 activation resulting in the liberation of AA and generation of Tx, however in agonist stimulated platelets these responses were elicited when the $[Ca^{++}]_i$ was significantly lower than predicted, indicating a synergism between Ca^{++} and other intracellular signal molecules in platelet activation (Hallam *et al.*1985; Rink and Hallam, 1984; Rink, 1988).

Platelet adhesion and aggregation are dependent on extracellular Ca^{++} as it is required for the binding of fibrin, von Willebrand factor and fibrinogen to their receptor sites on platelets (Siess, 1989). However, many agonist-induced platelet functional responses are also dependent on an increase in intracellular Ca^{++} in order to elicit a full response. Secretion of granule constituents is believed to be dependent on a rise in $[Ca^{++}]_i$, although granule release has been observed in platelets stimulated with phorbol esters, with no concomitant increase in $[Ca^{++}]_i$ suggesting that factors other than Ca^{++} are also involved in their release (Rink *et al.*1983). Many platelet enzymes including PLA_2, PKC and MLCKase are also dependent on an increase in $[Ca^{++}]_i$ for their activation, however, as with dense granule release the relationship between $[Ca^{++}]_i$ elevation and enzyme activation is not a simple one, but probably involves many other factors.

1.5.4 Annexins

Annexins (previously termed lipocortins/calpactins) are a widely distributed family of at least seven cytoskeletal proteins capable of binding phospholipids in the presence of Ca^{++} (Crumpton and Dedman, 1990; Russo-Marie, 1991).

Members of the annexin family share substantial sequence homology, with all except one member namely annexin VI, having a molecular weight of 32 - 38 KDa and consisting of 4 internal repeats of 70 amino acids (8 for annexin VI) (Russo-Marie, 1991).

The physiological role of annexins is unknown, although it has been suggested that they may be the mediators of glucocorticoid action (Flower and Blackwell, 1979; Flower, 1988) and that they could act as specific inhibitors of phospholipase A_2 (PLA_2) (Parente *et al.*1984; Wallner *et al.*1986). If annexins possess the ability to inhibit PLA_2 this would mean that they would be central to the control of prostaglandin, leukotriene and eicosanoid production and could thus in turn play a role in regulating the inflammation response. The ability of annexins to inhibit PLA_2 is however controversial (Davidson and Dennis, 1989) and may be due to substrate sequestration, moreover inhibition of PLA_2 has only been demonstrated *in vitro*.

The possibility that annexins may be important in intracellular signal transduction is also emerging with increasing evidence that annexins are cellular substrates for PTKases and PKC. Annexin I and II are substrates for the epidermal growth factor receptor and $pp60^{src}$ respectively, whilst annexins I,II,III,IV and VI are also substrates for PKC reviewed in Russo-Marie (1991). However, phosphorylation by these kinases has been found mainly *in vitro* with few examples of phosphorylation within whole cells (Coméra *et al.*1989; Khanna *et al.*1986; Gould *et al.*1986).

There is little information on the subtypes of annexins possessed by platelets or their possible functions in platelets (Touqui *et al.*1986; Crouch and Lapetina, 1986). Annexins are believed to function extracellularly (Flower and Blackwell, 1979; Russo-Marie, 1991), and platelets are known to release a variety of granule constituents upon activation, therefore it is possible that any annexins possessed by platelets may be released via the same route. The identity of annexins possessed by human platelets has been determined in this study, together with their ability to be released by several factors which induce granule release.

1.5.5 Protein phosphorylation

It is becoming increasingly evident that phosphorylation of proteins is a critical mechanism in the control of intracellular signalling in many different cell types including platelets. The main reasons for this are probably two fold, firstly, the addition of the phosphate group to a protein can substantially change its character, its conformation and its interaction with ions and with other proteins. Thus phosphorylation can make an inactive enzyme active or conversely an active enzyme inactive. The second important feature of protein phosphorylation is that due to the presence of phosphatases within cells the reaction is readily reversible and therefore can be closely controlled. Phosphorylation therefore controls the activation state of proteins which are responsible for the activation or inhibition of cellular responses.

The main mechanism by which receptor-coupled platelet activation is mediated is thought to be through protein phosphorylation. The calcium, calmodulin-dependent protein kinase, myosin light chain kinase, PKC and PTKase are several examples of protein kinases present in platelets. There are also signal transduction pathways in platelets which are linked to the inhibition of platelet functional responses and mediated by protein phosphorylation for example, an increase in cAMP caused by inhibitory platelet agonists such as prostacyclin or PGD_2 activates PKA. The first two phosphorylated proteins detected in agonist stimulated platelets were of 40-47 kDa and 20 kDa (Lyons and Shaw, 1980), now known to be the substrates of PKC and MLCKase respectively (Daniel *et al.*1984; Nishizuka, 1984).

1.5.6 Protein kinases in Platelets

1.5.6.1 Protein Kinase C

Protein kinase C (PKC) was discovered in 1977 by Nishizuka and colleagues and at that time was believed to be a single entity (Inoue *et al.*1977). However, subsequent molecular cloning and enzymological analysis has revealed the existence of at least seven isoenzymes of PKC (Nishizuka, 1988).

The putative endogenous activator of PKC is the physiologically relevant sn-1,2-diacylglycerol, a signal molecule produced by the hydrolysis of PtdIns(4,5)P_2, PtdIns(4)P and PtdIns (Nishizuka, 1984). Neither the sn-1,3-diacylglycerol enatiomer nor the 1,3-diglyceride diastereomer are capable of activating PKC. Activation of PKC is Ca^{++} and phospholipid, in particular phosphatidylserine, dependent (Nishizuka, 1986). Activated PKC phosphorylates its target proteins on their serine or threonine amino acid residues thereby regulating these proteins and their individual functions. In platelets two endogenous proteins of 40 kDa and 20 kDa are rapidly phosphorylated upon agonist-receptor coupling and their phosphorylation has been associated with granule release and platelet aggregation (Haslam and Lynham, 1977; Lyons and Shaw, 1980). The 20 kDa protein is myosin light chain (MLC) whilst the 40 kDa protein is the major substrate of PKC, although this protein has been sequenced and cloned its exact function still remains unknown (Touqui *et al.*1986; Connolly *et al.*1986). Once PKC is activated by DAG it is translocated from the cytosol to the membrane, where it is thought to be subjected to proteolytic cleavage by calpains to release a catalytically fully active fragment that is subsequently removed from the cell.

All isoenzymes of PKC are composed of a single polypeptide chain comprising several conserved and variable regions, the amino terminal half of all isoenzymes contain the conserved regions C_1 and C_2 which form the regulatory domain. The carboxy terminal half which contains C_3 and C_4 is the protein kinase domain. The conserved region C_3 also has an adenosine trisphosphate (ATP)-binding region, however, the sites involved in DAG and phospholipid binding have not yet been absolutely established (Nishizuka, 1988).

Although the functions of individual members of the PKC family have not yet been distinguished the roles attributed to PKC as a family in a variety of different cell types include involvement in secretion and exocytosis, modulation of ion

conductance, smooth muscle cell contraction, gene expression, down regulation of receptors, cell proliferation and regulation of signal transduction. In particular for platelets a role for PKC has been suggested in agonist-induced granule release, aggregation and AA release (Nishizuka, 1986).

1.5.6.1.1 Exogenous PKC activation and inhibition

It is now firmly established that phorbol esters such as 12-0-tetradecanoyl phorbol-13-acetate (TPA) which are well known tumour promoters directly activate PKC (Nishizuka, 1984; Castagna *et al.*1982). TPA has a DAG like structure and is able to substitute for DAG at extremely low concentrations. Similarly to DAG, TPA dramatically increases the affinity of the enzyme for Ca^{2+} which can result in full PKC activation without detectable mobilization of calcium (Rink *et al.*1983).

Phorbol esters and membrane permeant DAGs have been used in numerous studies to try and elucidate the role of PKC, however many of these studies have been carried out in either permeabilized cells, or cells where $[Ca^{++}]_i$ has been elevated using Ca^{++} ionophores (Halenda *et al.*1989; Halenda *et al.*1985). In addition, phorbol esters have different characteristics to endogenous DAG including their permeability and longevity, with phorbol esters causing a sustained activation of PKC. Therefore it is important to confirm that activated PKC really does possess the functions attributed to it from the extrapolations made from the results of experiments using, for example, phorbol esters as exogenous activators of PKC. One way of investigating the function of endogenously activated PKC is to use a PKC inhibitor. Some of the earliest PKC inhibitors described include chlorpromazine, dibucaine and other phospholipid interacting drugs including polymyxin B (Mazzei *et al.*1982) which inhibit PKC by competing with phospholipid. Later it was found that members of the isoquinoline sulfonamide family, particularly H-7, were capable of inhibiting PKC by competing with ATP for its binding site on PKC (Yamamoto and Hidaka, 1984). In 1985 the non-steroidal triphenylacrylonitrile (TPE) tamoxifen used in the treatment of breast cancer was shown to inhibit PKC (O'Brian *et al.*1985) and more recently was shown to be relatively selective for PKC (Spacey *et al.*1990). Depending on the structure of

the TPE, it inhibits PKC either at the regulatory or the catalytic domain (Bignon *et al.*1989). In order for the role of PKC to be unambiguous however, an inhibitor would need to be both potent and selective for PKC above other protein kinases. Until the discovery of staurosporine (Tamaoki *et al.*1986) and members of the same family including K252a and UCN01 (Ruegg and Burgess, 1989) all inhibitors of PKC had low potency and except tamoxifen were non-selective. Staurosporine and related compounds were found to be potent inhibitors of PKC but because they inhibited by competing with ATP for the ATP-cosubstrate site common to all protein kinases they had limited selectively for PKC (Ruegg and Burgess, 1989). Since the introduction of staurosporine other PKC inhibitors including amino acrides (Hannun and Bell, 1988), pseudo substrate analogues of PKC (Ricouart *et al.*1989) and calphostin C (Kobayashi *et al.*1989) have been produced which are thought to inhibit PKC at the catalytic and regulatory sites, at the substrate binding site and at the regulatory domain respectively. Recently several potent and selective inhibitors of PKC have been described (Davis *et al.*1989). Two of these compounds namely, Ro 31-7549/001 and Ro 31-8220/002, which although structurally based on staurosporine and inhibit PKC by competing with ATP for the co-substrate site, have been demonstrated to be selective for PKC over both protein kinase A and Ca^{++} calmodulin dependent kinase (Davis *et al.*1989) and have been used in this study in order to examine the role of endogenously activated PKC in the signal transduction pathway in PAF-stimulated rabbit platelets.

1.5.6.2 Myosin Light Chain Kinase

Following platelet activation and the ensuing increase in $[Ca^{++}]_i$, myosin light chain kinase (MLCKase) is activated resulting in phosphorylation of MLC. Once MLC has been activated by phosphorylation it associates with actin filaments of the cytoskeleton resulting in the contractile activity of platelets. Phosphorylated MLC is therefore involved in platelet morphological changes including shape change (Daniel *et al.*1984). Like PKC, MLCKase phosphorylates at either the

serine or threonine amino acid residues, its activation is Ca^{++} and calmodulin-dependent. Centralisation of granules before their release from the platelets is also associated with the movement of microfilaments surrounding the granules, phosphorylated myosin is believed to be a central component of the microfilament mass produced by this contractile wave (Fox, 1987). MLC is also phosphorylated by PKC although on a site distinct from that phosphorylated by MLCKase (Naka *et al.*1983).

1.5.6.3 Protein tyrosine kinase

Many phosphorylation events observed in platelets have been attributed to the role of PKC and MLCKase, both of which are serine/threonine specific kinases. However it is possible that recently discovered protein tyrosine kinases (PTKases) in platelets may also have a role in signal transduction.

Until recently PTKases were classified either as those whose genes had become part of oncogenic retroviruses or those associated with growth factor receptors (Hunter and Cooper, 1985). The most well studied PTKase oncogene product is probably $pp60^{v\text{-}src}$, coded by the transforming gene of the Rous sarcoma virus. Growth factor receptors which possess intrinsic PTKase activity include those for EGF, PDGF and also insulin and insulin-like growth factor 1 (IGF-1). These receptors possess intrinsic PTKase activity such that binding of the growth factor causes autophosphorylation of the receptor and the PTKase activity increases towards other substrates. Both groups of PTKases are therefore associated with either normal or abnormal cell growth (Hunter and Cooper, 1985). The cellular homolog of $pp60^{v\text{-}src}$ namely $pp60^{c\text{-}src}$ can also be activated by phosphorylation. In this case however a dephosphorylation of Tyr 527 results in a 10 fold increase in kinase activity coupled to an activation of its transforming activity.

However, more recently non-proliferating and terminally differentiated cells such as platelets (Tuy *et al.*1983) and post mitotic differentiated or differentiating neurons (Brugge *et al.*1985) have been found to possess PTKase activity,

suggesting therefore that PTKase may have some other role in cellular function distinct from cell growth or differentiation.

The first PTKase to be discovered in platelets was $pp60^{c\text{-}src}$ where it is expressed in exceptionally high levels of 0.2-0.4 percent of the total cellular protein (Golden *et al.*1986). The c-src gene which is homologous to the transforming gene v-src is highly conserved across the vertebrate species suggesting the product of the c-src gene has a role of fundamental importance. It is known that tyrosine phosphorylation is reversible and phosphotyrosine can be dephosphorylated with specific phosphatases, which although previously thought of as "house keeping" enzymes, may also play an essential role in intracellular regulatory mechanisms (Tonks and Charbonneau, 1989).

Tyrosine-specific phosphorylation of proteins represents the major kinase activity in human and rabbit platelet membranes intimating $pp60^{c\text{-}src}$ might serve a specific function in the platelet membrane. Compared to other cell types, platelet lysates contained 3-7 fold higher levels of $pp60^{c\text{-}src}$ than in brain or peripheral blood lymphocyte lysates, and 30-50 fold higher levels than in muscle, spleen, thymus, lymph node, bone marrow or erythrocyte lysates (Golden *et al.*1986). Indeed, the precursor cell of platelets, the megakaryocyte, also possesses elevated levels of the $pp60^{c\text{-}src}$ specific kinase activity (Golden *et al.*1986).

It has only been relatively recently demonstrated that activation of platelets with agonists including thrombin and collagen induce rapid tyrosine phosphorylation on a number of proteins by stimulating one or more PTKase in intact human platelets (Nakamura and Yamamura, 1989; Ferrell and Martin, 1988).

1.5.6.4 Protein phosphorylation associated with inhibition of platelet activation

Inhibitory agonists for example PGI_2, PGD_2 and adenosine suppress platelet responsiveness by activating adenylate cyclase and increasing the concentration of cytosolic cAMP which activates cAMP dependent kinase (protein kinase A) to phosphorylate its target proteins. The increase in cAMP levels is transient, with

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platelets possessing cyclic nucleotide phosphodiesterases specific for cAMP (Crawford and Scrutton, 1987).

The site of action of PKA has not been identified, but one of the mechanisms by which cAMP regulates platelet functions is through regulating the level of $[Ca^{++}]_i$ (Feinstein *et al.*1985). Cyclic AMP exerts two types of effect on $[Ca^{++}]_i$, firstly it inhibits elevation of $[Ca^{++}]_i$ induced by a number of different agonists and secondly it stimulates resequestration of $[Ca^{++}]_i$ which has already been elevated reviewed in Feinstein *et al.* (1985). It has been suggested that activated PKA may promote resequestration of Ca^{++} into the endoplasmic reticulum by increasing the Ca^{2+} Mg^{2+}-ATPase activity possessed by that membrane, believed to be one of the mechanisms of Ca^{++} homeostasis in platelets. However, other mechanisms must also play a role as sequestration of Ca^{++} could only explain a dampening of the response but not a complete prevention of agonist-induced $[Ca^{++}]_i$ elevation. It has also been suggested that increasing cAMP levels may inhibit agonist-induced PtdIns(4,5)P_2 hydrolysis and therefore suppress elevation of the two second messengers Ins(1,4,5)P_3 and sn-1,2-DAG (Rittenhouse-Simmons, 1979; Knight and Scrutton, 1984; Siess, 1989). Moreover, the finding that an increased level of cAMP totally prevents agonist-induced $[Ca^{++}]_i$ elevation suggests cAMP is able to exert an effect on Ca^{++} influx across the plasma membrane, this may be due to a cAMP-induced inhibition of agonist binding as has been demonstrated in thrombin-stimulated platelets (Lerea *et al.*1987). However, in PAF-stimulated platelets a much lower level of cAMP is required to inhibit aggregation and secretion than to cause inhibition of PtdIns(4,5)P_2 hydrolysis or $[Ca^{++}]_i$ elevation suggesting a mechanism distal to inhibition of PLC activity (Bushfield *et al.*1985). Indeed, recently it has been demonstrated that activation of PKA inhibits platelet aggregation at steps distal to PKC and Ca^{++}-dependent protein phosphorylation (Siess and Lapetina, 1989). Activation of platelets by excitatory agonists is not believed to be caused by decreasing cAMP levels, although several agonists, for example ADP, adrenaline and TxA_2 can decrease cAMP elevation induced by an inhibitory agonist such as PGD_2 reviewed in Feinstein *et al.* (1985).

Exposure of platelets to EDRF (nitric oxide) released from endothelial cells (Palmer *et al.*1987) causes an activation of guanylate cyclase and increases cyclic GMP. As a result of cGMP elevation platelet responses are inhibited, however, little is known of the mechanisms by which cGMP depresses platelet responsiveness or indeed whether it involves protein phosphorylation. Moreover, excitatory agonists such as ADP, collagen and thrombin also cause an increase in cGMP levels and it is therefore possible that it may act as a negative feedback pathway. Its mechanisms of action are however, separate from those used by cAMP as there are differences in the pattern of responses inhibited by the two cyclic nucleotides, for example, adhesion to collagen is markedly inhibited by an increase in cGMP but affected little by increases in cAMP (Radomski *et al.*1987a).

1.6 AIMS

To further elucidate the signal transduction pathway of PAF-stimulated platelets and to investigate the role of protein phosphorylation in platelet signal transduction, in particular, to investigate the role of protein kinase C and tyrosine kinase in PAF-induced platelet activation.

1. To investigate the modulatory role of PKC in PAF, ionomycin and arachidonic acid-induced platelet functional responses.

It was proposed to examine the role of protein kinase C in platelets by investigating the effect of both activation and inhibition of PKC on the functional responses of platelets induced by three different mechanisms. Platelets were stimulated with an agonist (platelet-activating factor), by direct elevation of cytosolic Ca^{++} (ionomycin) or by arachidonic acid (AA) which is metabolised directly into measurable thromboxane B_2. This study was designed to elucidate where in the signal transduction pathway PKC-induced protein phosphorylation may have a regulatory role.

2. To characterise signal molecule elevation in PAF stimulated platelets and to investigate the role of PKC on signal molecule production

Few studies using intact cell preparations have been performed to evaluate the relationship between the signal molecules $[Ca^{++}]_i$, Ins(1,4,5)P_3 and sn-1,2-DAG. In this study therefore the aim was to determine the temporal and dose-dependent relationships of PAF-induced elevations in $[Ca^{2+}]_i$ to changes in the levels of Ins(1,4,5)P_3 and sn-1,2-DAG in intact rabbit platelets. As an alternative experimental approach it was proposed to use the potent, although non-specific, PKC inhibitor staurosporine to examine the role of endogenously activated PKC in the regulation of receptor-mediated changes in the intracellular concentrations of Ca^{++}, Ins(1,4,5)P_3 and DAG.

3. To investigate the role of endogenously activated PKC in platelet functional responses using selective inhibitors of PKC.

Throughout the course of this study there have been an increasing number of reports describing the development of selective inhibitors of PKC. These selective inhibitors included calphostin C (Kobayashi *et al.*1989) and the Roche compounds Ro 31-8220/002 and Ro 31-7549/001 (Davis *et al.*1989). Using these compounds it is likely that a more accurate insight could be gained into the role of endogenously activated PKC than when using a non-selective inhibitor. The aim was therefore to investigate the role of endogenously activated PKC using these newly developed PKC inhibitors and to compare their effects on platelet functional responses in PAF-stimulated platelets to the findings for staurosporine.

4. To determine the identification and location of annexin isoforms in human platelets

The ability of annexins to inhibit PLA_2 was the first functional response attributed to them in which they were linked to signal transduction. Annexins are believed to be phosphorylated by PKC and PTKase and therefore it is possible that they may be involved in the regulation of phospholipid hydrolysis and be regulated themselves by their state of phosphorylation. The identity of annexins in platelets has not been determined and therefore the aim of this study was to determine the identification and location of annexin isoforms in human platelets, and to determined whether they could be released from the granules upon platelet stimulation.

5. To characterise tyrosine phosphorylation of proteins in PAF-stimulated platelets and to investigate the role of endogenously activated tyrosine kinase(s) in platelet signal transduction using the tyrosine kinase inhibitor genistein.

It has only been relatively recently that the presence of tyrosine kinase(s) in terminally differentiated cells such as platelets has been recognised. Their role is usually associated with cell growth and proliferation, however in platelets due to the rapidity of tyrosine phosphorylation of protein substrates upon receptor-agonist coupling it is possible that they may have a role in signal transduction. In this part of the study the aim was to determine the temporal and dose-dependent relationship of PAF-induced elevation of tyrosine phosphorylated proteins induced by tyrosine kinase activity. It was also proposed to use an inhibitor of tyrosine kinase(s) in an attempt to identify the role of endogenously activated tyrosine kinase(s) both in signal molecule production and platelet functional responses.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 GENERAL REAGENTS

Albumin Bovine (fraction V, protease-free); Arachidonic Acid (sodium salt); ARG-GLY-ASP-SER (acetate salt), Adenosine 5'-trisphosphate (ATP, disodium salt); Coomassie Brilliant Blue R-250; Dextran; 3,3'-diaminobenzidine (DAB); Diethlenetriaminepenta-acetic acid; ethylenediamine-tetraacetic acid (EDTA); ethylene glycol-bis[ß-aminoethyl ether] N,N,N',N'-tetraacetic acid (EGTA); Gelatin (type A: from porcine skin); N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); Human-α-thrombin; Imidazole; Indomethacin; High Molecular weight markers (SDS 6H);O-phospho-DL-serine (DL-2-amino-3-hydroxypropanoic acid 3-phosphate); O-phospho-DL-tyrosine (DL-3-[Hyroxyphenul]alanine 4'-phosphate); O-phospho-DL-threonine (DL-2-Amino-3-hydroxybutanoic acid 3-phosphate); Sodium dodecyl sulphate (lauryl sulphate); 12-O-tetradecanoylphorbol-13-acetate (TPA); Sodium ethylmercurithiosalicylate (thiomersal); Tris[hydroxymethyl]aminomethane (TRIZMA BASE); polyoxyethylenesorbitan monolaurate (Tween 20) and TxB_2 standard were all purchased from Sigma Chemical Company Ltd., Poole, Dorset, UK.

Acetic acid (AnalaR); Acrylamide (Electran); Ammonium peroxodisulphate (Electran); Calcium Chloride (AnalaR); Charcoal ('Norit GSX'); Digitonin (AnalaR); D-Glucose (AnalaR); Glycerol (AnalaR); Hydrogen Peroxide solution (Analar); Potassium chloride (AnalaR); Magnesium chloride hexahydrate (AnalaR); 2-mercaptoethanol (Biochemical); NN'-methylenebisacrylamide (Electran); Ponceau S (Electran); Sodium dihydrogen orthophosphate; Sodium hydroxide; Tri-sodium citrate (AnalaR) were all purchased from BDH Ltd., Bristol, Avon UK. Chloroform (HPLC solvent); Hydrogen chloride; Methanol (HPLC solvent); Heparin (Heparin Sodium, mucous) and Sodium chloride (Analytical Reagent) were purchased from Fisons, FSA Laboratory Supplies, Loughborough, Leicester, UK. Ionomycin (free acid) was purchased from Calbiochem Company, Novabiochem (UK) Ltd.,

Nottingham, UK and PAF (1-O-Octadecyl-2-O-acetyl-sn-glycero-3-phosphocholine) was from Bachem Bioscience Inc. Bubendorf, Switzerland. Staurosporine was purchased from Fluka Chemicals, Glossop, Derbyshire UK and Genistein was obtained from Gibco BRL, Uxbridge, Middlesex, UK. Bromophenol Blue and N,N,N',N'-Tetramethylethylenediamine (TEMED) were purchased from Bio-Rad, Hemel Hempstead, Hertfordshire, UK.

5-[^{14}C]Hydroxytryptamine creatinine sulphate (54 mCi/mmol, 50 μCi/ml), [^{32}P]Pi (10 mCi/ml), [^{32}P]-ATP (3 Ci/mmol) and [^{3}H]PAF (80 Ci/mmol, 250 μCi/ml) were purchased from Amersham International, Aylesbury, Bucks, UK. [^{3}H]Thromboxane B_2 (125 Ci/mmol, 50 nCi/ml) was obtained from NEN Research Products, DuPont (UK) Ltd., Stevenage, Hertford, UK.

Inositol (1,4,5)-Trisphosphate assay system (TRK 1000) and the sn-1,2-Diacylglycerol assay system (RPN 200) were purchased from Amersham Life Sciences, Aylesbury Bucks, UK. Fura-2-acetoxymethyl ester, Fluo-3-acetoxymethyl ester, Fura-2 free acid, Fluo-3 free acid and pluronic acid (F-127) were purchased from Molecular Probes, Eugene, Oregon, USA. X-ray film was obtained from Fuji Photo Film Co.Ltd., X-ray film developer (kodalith liquid developer) and fixative were purchased from Kodak, Hemel Hempsted, England. Thin layer chromatography plates (silica gel 60 HPTLC aluminium, 20 x 20 cm) were purchased from E.Merck, Darmstadt, FRG. Nitrocellulose paper (0.45 μm) was obtained from Schleicher & Schuell, Dassel, Germany, whilst Whatman 3MM filter Paper was purchased from Whatman Laboratory Sales, Maidstone, Kent, UK. Sagital was purchased from May & Baker Laboratory Products, Rhone-Poulenc, Mancester, UK and Hypnorm was purchased from Janssen Pharmaceuticals Ltd., Oxon, UK.

2.1.2 SOLUTIONS

Aggregation inhibitor

ARG-GLY-ASP-SER was dissolved (50 mg/ml) in normal saline as required.

Fluorescent Probes

Fura-2-AM, fura-2 free acid, fluo-3-AM and fluo-3 free acid were stored solid and dry at -20°C. Stock solutions of fura-2-AM and fluo-3-AM were dissolved in anhydrous DMSO at a concentration of 5 x 10^{-3}M and stored in darkness at -20°C. Stock solutions of fura-2 free acid and fluo-3 free acid were dissolved in distilled water, subaliquoted and stored at -20°C. Pluronic acid (F-127) was dissolved 1:3 (W:W) in DMSO by warming at 37°C for 30 minutes it was then stored at room temperature.

PAF antagonist

WEB 2086 a gift from Dr H. Huer, Boehringer, Ingelheim, Germany was dissolved at a concentration of 10^{-3}M in PBS (37°C) on the day required.

PKC activators

12-O-tetradecanoyl 13-phorbolacetate was dissolved in dry acetone (1 mg/ml) and stored at -20°C, further dilutions were made in PBS.

1,2-dihexanyl-sn-diacylglycerol was a gift from Dr L. Garland, Wellcome Laboratories, Beckenham, Kent. A stock solution was stored in chloroform, this was aliquoted and dried under nitrogen and then dissolved in ethanol (50 mg/ml). Further dilutions were made in normal saline.

PKC inhibitors

Calphostin C was a gift from Dr.T.Tamaoki, Kyowa Hakko Kogyo Co., Ltd. Toyko, Japan. A stock solution at a concentration of 10^{-2} M was dissolved DMSO and stored at -20°C. The stock was diluted to 10^{-3}M in ethanol and further dilutions were made into HBT.

Ro 31-7549/001 and Ro 31-8220/002 were gifts from Dr. J S Nixon, Roche, Herts. They were stored solid and dry at -20°C. When required they were dissolved at 10^{-2}M in DMSO, further dilutions were made in PBS.

Staurosporine was dissolved in 70% ethanol and stored at -20°C in darkness, further dilutions were made in PBS.

Platelet stimulants

Arachidonic acid (free acid) - A stock solution was dissolved in absolute ethanol (5 x 10^{-2} M) and stored under nitrogen and in the dark at -20°C.

Ionomycin - A stock solution of 5 x 10^{-4}M was dissolved in DMSO and stored at 4°C.

PAF (1-O-Octadecyl-2-acetyl-sn-glycero-3-phosphorylcholine) - A 10^{-2}M stock solution was dissolved in chloroform:methanol (4:1 v/v) and stored at -20°C. Subaliquots (10 μl) were taken, dried under nitrogen and then made up to 1 ml in PBS containing 2.5 mg/ml BSA to give a final concentration of 10^{-4}M PAF. The aliquots were then stored at -20°C.

Human-α-Thrombin - The stock solution containing 60 NIH units/ml was stored in 0.1 M Triethanolamine, 0.1M NaCl, 0.1% polyethyleneglycol (6000 MW), pH 7.8, at -20°C.

Tyrosine Kinase inhibitor

Genistein was stored solid and dry at -20°C. As required it was dissolved at a concentration of 10 mg/ml in DMSO, further dilutions were made in distilled H_2O.

2.2 METHODS

2.2.1 ANIMALS

New Zealand white rabbits 2.5 - 3 Kg either male or female were used. They were caged either singly or doubly or in groups in floor pens and food and water were freely available.

2.2.2 BLOOD COLLECTIONS

Blood was collected by either of two methods:-

Method 1

Rabbits were anaesthetized by an intravenous injection of anaesthetic into the ear vein of 2 ml/Kg of 20 mg/ml pentobarbitone (Sagital), giving a final concentration of 40 mg/Kg rabbit. Due to the variability in response experienced between individual rabbits the anaesthetic was administered slowly and the degree of anaesthesia assessed until the required depth was attained.

The rabbits were then bled by cannulation of the carotid artery and the blood collected into 3.2% trisodium citrate (9:1, v/v). The blood and anticoagulant were then gently mixed.

Method 2

New Zealand White rabbits 3.5 - 4 Kg were sedated with an intramuscular injection of 0.25 ml/Kg of Hypnorm (0.315 mg fentanyl citrate/ml and 10 mg fluanisone/ml). After sedation (about 30 minutes) a rabbit was bled up to 35 mls from the ear artery using a 19 gauge needle and the blood was collected into 3.2% trisodium citrate (9:1, v/v). The blood and anticoagulant were gently mixed.

2.2.3 PREPARATION OF WASHED RABBIT PLATELETS

2.2.3.1 Prostacyclin

Prostacyclin (PGI_2) a gift from Dr Whittle, Wellcome Laboratories, Kent, was stored solid and dry at -20°C. Stock aliquots were dissolved in 0.5 M Tris-HCl (pH 10.5) and were stored at a concentration of 40 μg/40 μl at -20°C. Immediately prior to use an aliquot was thawed and diluted as required in 0.01M Tris-HCl (pH 10.5) and kept on ice.

2.2.3.2 HEPES Buffered Tyrode's solution

	(per litre)
10 mM HEPES	(2.4 g)
145 mM NaCl	(8.4 g)
5 mM KCl	(375 mg)
1 mM $MgCl_2$	(200 mg)
0.5 mM Na_2HPO_4	(101 mg)

The constituents were dissolved in 900 ml of distilled H_2O and the pH adjusted to 7.4 with NaOH and the total volume made up to 1 litre. On the day of an experiment 5.5 mM glucose and 0.25% bovine serum albumin was added to the required volume of buffer.

2.2.3.3 Preparation of platelet rich plasma

Washed platelets were prepared essentially as described by Poll *et al.* (1986) for human platelets, using a modification of the method of Blackwell *et al.* (1982b). After collection rabbit blood was centrifuged immediately at 185 x g (rt) for 10 minutes. The top layer of platelet rich plasma (PRP) was removed from the lower red blood cell layer.

2.2.3.4 Washed platelet preparation

PGI_2 (300 ng/ml) was added to the PRP which was gently mixed and then centrifuged at 1256 x g (rt) for 15 minutes to precipitate a platelet pellet. The plasma was removed from the platelet pellet which was gently resuspended into HBT using a pastette, 100 ng/ml of PGI_2 was then added to the suspension. The platelet suspension was centrifuged at 1256 x g (rt) for 15 minutes to precipitate a platelet pellet.

2.2.3.5 Preparation of washed human platelets

Human venous blood was collected by forearm venepuncture from normal volunteers (who denied having taken any medication for the previous 14 days) into 1 unit heparin/ml of blood. Washed human platelets were prepared as described for rabbit platelets in sections 2.2.3 using HBT containing 1 mM $CaCl_2$.

2.2.4 GENERAL EXPERIMENTAL CONDITIONS

Platelets were allowed to 'recover' for 90 minutes after they had been resuspended in PGI_2 free HBT to allow increased cAMP levels induced by PGI_2 to decline to basal levels and full functional activity to return. Except where stated $[Ca^{++}]_e$ was adjusted to 1 mM before experimentation. In all experiments platelets were allowed to equilibrate to 37°C before being activated.

2.2.5 PLATELET COUNTING

Platelet counts were made using an electronic particle counter (coulter counter) calibrated for platelet size. Platelets were diluted (1:1000) with isoton II azide free diluent and the total platelet count assessed. The coulter counter measured the number of platelets in 50 μl therefore in order to determine the number of platelets/ml the count on the digital readout was multiplied by 20 x 1000.

2.2.6 AGGREGOMETRY

The technique used to examine platelet aggregation in this study was the turbidimetric method described by Born (1962). This method uses an optical aggregometer to detect changes in light transmission through the platelet suspension in response to an agonist. Platelet shape change in response to an agonist will cause a decrease in light transmission whilst aggregation causes an increase in transmission.

2.2.6.1 Platelet preparation

Platelets were prepared as described in section 2.2.3 and the washing procedure repeated once more in order to wash the platelets twice before use. After the final centrifugation the platelets were resuspended to a concentration of 2 x 10^8/ml.

2.2.6.2 Aggregation assay

Aggregation assays were carried out in a Chronolog dual channel ionized Ca^{++} aggregometer (Born, 1962). The calibration of the light transmission was carried out by making a 10 fold dilution of washed platelets (2 x 10^7/ml), the light transmission through this suspension represented maximum light transmission termed 100%. Basal (0%) light transmission was obtained using 2 x 10^8 platelets/ml. The platelets were stirred at 900 rpm for the duration of the experiment. Platelet shape change was measured as a decrease in light transmission whilst aggregation was monitored as an increase in light transmission and recorded using a two channel potentiometric chart recorder (chart speed 3 cm/min). Aggregometry traces were quantified by measuring the rate of aggregation.

2.2.7 DETERMINATION OF CYTOSOLIC CALCIUM

2.2.7.1. Loading with cell permeant Fura-2-acetoxymethyl ester

Platelets were prepared as described in section 2.2.3. The platelet pellet was resuspended to 8 x 10^8 platelets/ml and 0.5 μl/ml (2.5 μM final concentration) fura-2-AM added. The platelets were then incubated at 37°C for 30 minutes. The platelets were occasionally agitated gently to aid fura-2-AM loading, whilst the BSA contained in the HBT aided dye dispersal. The platelet suspension was then washed three times in HBT to remove excess fura-2-AM and the platelet count adjusted to 2 x 10^8/ml. Aliquots of platelets (1 ml) were dispensed into fluorimeter cuvettes and changes in $[Ca^{++}]_i$ fluorescence were recorded using an Aminco Bowman Spectrophotofluorimeter (ex = 339 nm; emission = 500 nm; 4 nm slit width) fitted with a thermostatted cuvette compartment holder with stirring attachment.

2.2.7.2 Calibration

The Ca^{++}-fura-2-fluorescence was calibrated as previously described (Pollock *et al.*1986; Grynkiewicz *et al.*1985). At the beginning of an experiment and every 30 minutes thereafter, the Ca^{++}-fura-2-fluorescence was calibrated by measuring the maximal fluorescence signal (Fmax) and the minimum fluorescence signal (Fmin). The Fmax was achieved by lysing platelets in the presence of 1 mM $[Ca^{++}]_e$ with 50 μM digitonin (taken from a stock of 1 mM digitonin in 70% ethanol). Digitonin caused complete lysis of the platelets and released intracellular fura-2 which then had access to excess $[Ca^{++}]_e$ and therefore caused maximal possible fluorescence. Subsequently adjusting the cell lysate pH to >8.5 with NaOH and chelating $[Ca^{++}]_e$ with 6 mM EGTA the fluorescence signal in Ca^{++} free medium will cause the fluorescence to drop to minimum [Fmin]. To assess the amount of fluorescence due to extracellular non-loaded or leaked fura-2, 1 mM $NiCl_2$ was added to 1ml of platelets. The quench in the fluorescence observed was due to extracellular fura-2-AM as $NiCl_2$ is not cell permeant (Hallam and Rink, 1985b).

Changes in fluorescence were recorded on a potentiometric chart recorder (Rikadenki) which was linked up to the fluorimeter.

In order to convert the fluorescence values (F) from the chart recording into cytosolic calcium concentration ($[Ca^{++}]_i$) the fluorescence values in arbitrary units were put into the equation:

$$\frac{F(-Ni) - Fmin}{Fmax - F\text{-}(Ni)} \times Kd = [Ca^{++}]_i \text{ (nM)}$$

Kd = the dissociation constant of the Ca^{++}-fura-2 complex which is equal to 224 nM (Grynkiewicz *et al.*1985). The dissociation constant of the Ca^{++}-fluo-3 complex is equal to 400 nM (Kao *et al.*1989).

The results for Ca^{++} studies have either been given as maximal $[Ca^{+}]_i$ elevation (nM) or as the $T_{1/2}$, which is the time taken for $[Ca^{++}]_i$ to decline to 1/2 maximal above basal levels. A representative trace of $[Ca^{++}]_i$ elevation is given in figure 5, demonstrating the concepts of maximal $[Ca^{++}]_i$ elevation and $T_{1/2}$. The $T_{1/2}$ was calculated by working out the maximal $[Ca^{++}]_i$, and the basal $[Ca^{++}]_i$ then:-

$$\frac{\text{maximal } [Ca^{++}]_i - \text{basal } [Ca^{++}]_i}{2} = \text{the } [Ca^{++}]_i \text{ at } T_{1/2} ([Ca^{++}]_i T_{1/2}) \text{ termed Y}$$

the arbitrary fluorescence reading at $T_{1/2}$ was termed B and put into the equation

$$\frac{B - Ni - Fmin}{Fmax - [B - (-Ni)]} \times 224 = Y$$

then:-

$$B - Ni - Fmin = \frac{YFmax + Yni - YB}{224}$$

then:- $$B + YB = \frac{YFmax + YNi}{224} + Ni + Fmin$$

this will then give an arbitrary number on the trace from which the measurement of time from peak $[Ca^{++}]_i$ to $T_{1/2}$ can be taken.

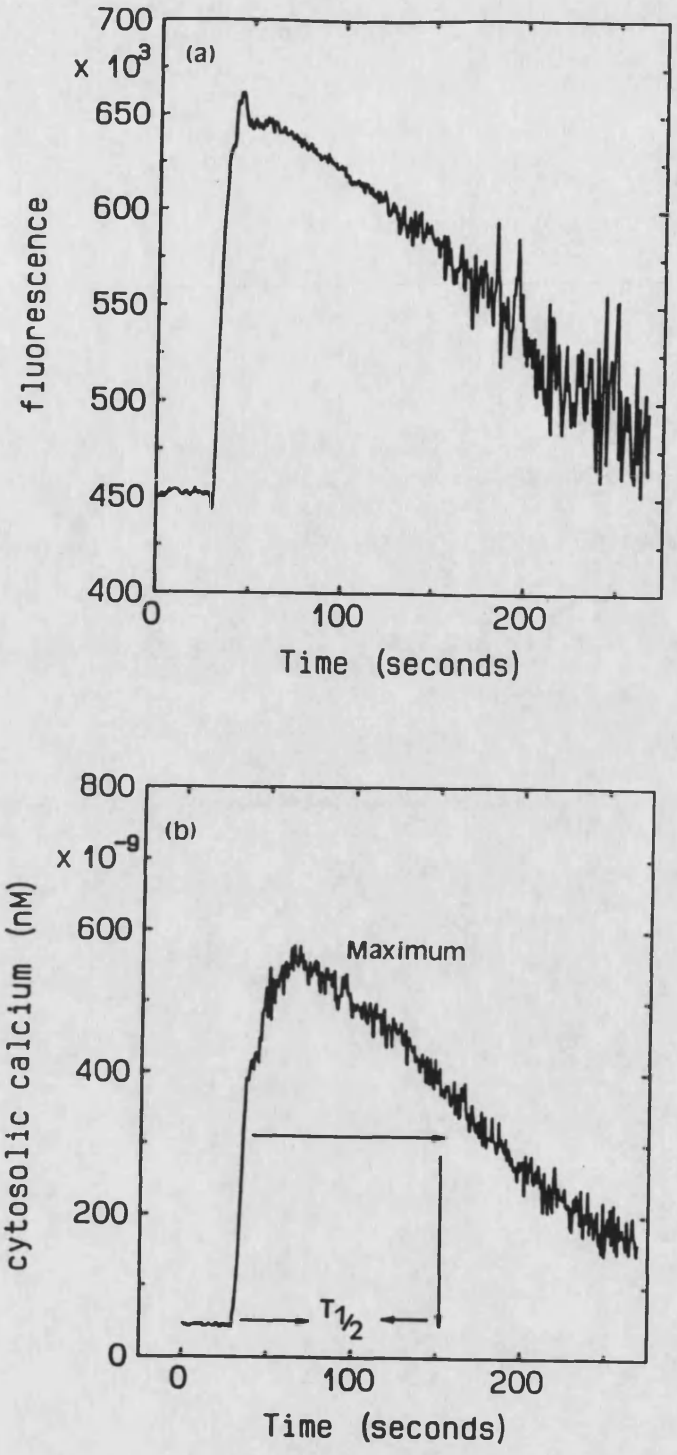

Figure 5 Cytosolic Ca^{++} elevation demonstrated as fluorescence output from fura-2 loaded platelets and as $[Ca^{++}]_i$ (nM). (a) Representative trace of fluorescence output from fura-2-loaded platelets stimulated with 10 nM PAF (b) the same trace as (a) but the fluoresence has been converted to $[Ca^{++}]_i$ (nM). Panel (b) also demonstrates the concepts of maximal cytosolic calcium elevation and $t_{1/2}$ (the time taken for Ca^{++} levels to decline to 1/2 maximal above basal).

2.2.7.3 The fluorescent Ca^{++} indicator fura-2-AM

The fluorescent Ca^{++} indicator fura-2-acetoxymethyl ester was first described in 1985 by Tsien and colleagues (Grynkiewicz *et al.* 1985). The fluorescence of fura-2 is about 30 times brighter than its predecessor quin-2 (about 6 times the absorbance and 5 time the quantum yield) therefore a much lower concentration of fura-2 is needed to adequately load cells. Whereas quin-2 requires about 1 mM of trapped probe for an adequate signal fura-2 only requires between 10 to 50 μM. The acetoxymethyl ester is membrane permeant, but once inside the cells is hydrolysed by non-specific esterases giving formaldehyde and acetic acid and liberating the free chelator which is not membrane permeant and therefore concentrates within the cell. The lower concentration of fura-2 compared to quin-2 results in less acidification and toxicity by the cells caused either by the chelator, acetic acid or formaldehyde which are released by intracellular enzymatic hydrolysis of the acetoxymethyl (AM) esters. Fura-2 therefore also causes less buffering of Ca^{++} than quin-2 and subsequently less damping of the Ca^{++} transient.

The maximum absorbance of fura-2 shifts from 362 nm for the Ca^{++}-free chelator to about 335 nm for the Ca^{++} saturated form with the highest dynamic range for Ca^{++} measurement being obtained by comparing the ratio of maximal chelator fluorescence at 510 nm with exc near 340 & 380 nm. However it is important that all four AM esters on the chelating acetic acids are hydrolysed in order to achieve Ca^{++} sensitive emission.

2.2.7.4 Concentration of intracellular fura-2

A standard curve of fluorescence output against concentration of cell impermeant fura-2 free acid (10 nM - 100 nM) was performed in the presence of 1 mM $[Ca^{++}]_e$. Platelets loaded with fura-2-am as described in section 2.2.7.2 were counted and then lysed with 50 μM digitonin. The suspension was centrifuged, the supernatant removed and the fluorescence in the supernatant measured in the

presence of 1 mM $[Ca^{++}]_e$. The concentration of fura-2 in the supernatant was then read from the standard curve. On the basis that the intracellular volume of 10^9 platelets is equal to 10 μl (Rittenhouse and Sasson, 1985) it was then possible to calculate the intracellular concentration of fura-2 in the platelet preparation. The intracellular concentration of fura-2 was found to be between 10 - 20 μM, which is at the lower end of the recommended range, therefore reducing the problem of artifacts due to incomplete hydrolysis of fura-2-AM, and reducing the Ca^{++} buffering effects of fura-2.

2.2.7.5 Fluo-3-AM

Fluo-3-AM (Minta *et al.*1989) is a new long wavelength indicator of intracellular Ca^{++}. Platelets were loaded with fluo-3-AM essentially as described for fura-2. However, before adding fluo-3 to the platelets it was first diluted in 1 ml of HBT to which 5 μl pluoronic F-127 had been added. The mixture was then added to the platelets (8 x 10^8/ml) to give a final concentration of 5 μM fluo-3. The platelets were then incubated for one hour at 37°C before washing as described for fura-2 loaded platelets. Addition of pluronic F-127 (which is a low ionic, low toxicity detergent) during incubation of fluo-3 increases solubilization and dispersal of the dye. Aliquots of platelets (2 ml) were dispensed into fluorimeter cuvettes and changes in $[Ca^{++}]_i$ fluorescence measured using a PTI dual excitation spectrophotometer (ex 506 nm; emission 526 nm; 4 nm slit width).

2.2.8 DETERMINATION OF DENSE GRANULE RELEASE IN PLATELETS

2.2.8.1 5-HT uptake into granules

Platelets accumulate 5-hydroxytryptamine (5-HT, serotonin) against a concentration gradient by several mechanisms into their dense granules (Pletscher, 1968). The assay used in this study to determine dense granule release has taken advantage of this accumulation of 5-HT by using ^{14}C-5-HT which is accumulated upon incubation into the granules and then released upon platelet activation with an excitatory agonist to act as a marker for release of dense granule constituents.

2.2.8.2 Platelet preparation

Platelets were prepared as described in section 2.2.3. The platelet pellet was suspended to 8 x 10^8 platelets/ml and incubated with 200 nCi/ml 5-[^{14}C]-HT at 37°C for 30 minutes. The platelets were then washed three times to remove extracellular [^{14}C]-5HT and the final platelet count was adjusted to 2 x 10^8/ml. Aliquots of platelets (1 ml) were dispensed into eppendorf tubes and treated as described for individual experiments. [^{14}C]-5HT release was terminated 3 minutes after platelet activation by removing a subaliquot of 400 μl into ice-cold tubes containing 40 μl of 100 mM EDTA and 30 μM indomethacin. The samples were then centrifuged at 12000 x g (2 minutes) and 2 x 100 μl samples of the supernatant removed. The amount of [^{14}C]-5-HT was determined by liquid scintillation spectroscopy counting each sample for 3 minutes.

Percentage release of 5-HT from platelets (Ts) was calculated using the following equation (Holmsen and Dangelmaier, 1989):-

$$\% \text{ 5-HT release} = \frac{Ts(dpm) - Bo(dpm)}{Tc(dpm) - Bo(dpm)} \times 100$$

where Tc = the total dpm in a sample of 100 μl of uncentrifuged platelets

where Bo = the dpm due to extracellular [^{14}C]-5HT in untreated, centrifuged platelets. [^{14}C]-5HT release was terminated 3 minutes after platelet activation as at this time, for all platelet stimulants used, 5-HT release had reached maximal and further incubation caused no further release of ^{14}C-5HT.

2.2.9 THROMBOXANE GENERATION

2.2.9.1 Materials

Anti-TxB_2 antibody was a gift from Dr.F.Carey, ICI, Macclesfied, UK.

Buffers

Buffer A (for dilution of antibody and [^{3}H]TxB_2)

$NaH_2PO_4.H_2O$ 6.2 g

Thiomersal 0.25 g

BSA fraction V 5.1 g

pH to 7.4 with NaOH, made up to 1 L with distilled H_2O

Buffer B (for dilution of samples and standards)

NaCl 1.8 g

made up to 200 mls with Buffer A_1

[^{3}H]TxB_2 label was stored as a stock solution in ethanol (10 μCi/ml). Further dilutions were made by adding 100 μl [^{3}H]TxB_2 to 20 mls of buffer A to give a final count of 10,000 dpm/100 μl. TxB_2 standard was made to a concentration of 20 ng/ml in PBS buffer and anti-TxB_2 antibody was diluted 1:500 in Buffer A.

Dextran coated charcoal

0.5 % w/v dextran T70

1% (w/v) charcoal GSX

made in buffer A (4°C, continually stirred).

2.2.9.2 Preparation of samples

Platelets were prepared as described in section 2.2.3 and washed twice before suspension to a final count of 2 x 10^8 pl/ml. Aliquots of platelets (1 ml) were dispensed into eppendorfs, and treated as described for individual experiments. Platelet activation was terminated at equilibrium (3 minutes) by removing a 400 μl subaliquot of platelets into an ice-cooled tube containing 40 μl of 100 mM EGTA and 30 μM indomethacin. The samples were then centrifuged at 12000 x g (2 minutes), and the supernatant removed and stored at -20°C for radioimmunoassay (Johnston *et al.*1984).

2.2.9.3 Radioimmunoassay

Serial dilutions of the TxB_2 standard from 1 to 1000 pg/100 μl and a range of dilutions of each of the samples to be assayed were made in Buffer B. The samples (or standards), [^{3}H]TxB_2 and anti-TxB_2 antibody were added to each eppendorf respectively in a ratio of 1:1:2. In order to calculate TxB_2 generation the total count (Tc), total [^{3}H]TxB_2 binding to the antibody (Tb) and non-specific binding (Nsb) were also required.

After all additions had been made, the tubes were mixed and incubated at 4°C for 18 - 24 hours. Dextran coated charcoal (the same volume as the TxB_2 antibody) was then added to each tube except the Tc and after incubation at 4°C for 15 minutes the tubes were centrifuged at 2000 x g for 15 minutes (4°C). The supernatant from each was decanted into a scintillation vial to which 4 mls of scintillant was added and the [^{3}H]TxB_2 in each was determined by liquid scintillation spectroscopy with each sample counted for 3 minutes.

From the counts a standard curve was constructed using % binding which was calculated by

$$\%\ [^3H]\ \text{binding} = \frac{\text{dpm(standards)} - \text{Nsb}}{\text{Tb} - \text{Nsb}} \times 100$$

The amount of TxB_2 for each sample (pg/100 μl) was then determined from the standard curve and converted to pmol/10^8 platelets. In common with dense granule release, TxB_2 generation was terminated at 3 minutes after platelet activation. At this time TxB_2 generation had reached maximal for all of the platelet activators being used, and further incubation caused no measurable increase in TxB_2 generation.

2.2.10 INOSITOL (1,4,5)-TRISPHOSPHATE

2.2.10.1 Sample preparation

Platelets were prepared as described in section 2.2.3, washed twice and the final platelet count adjusted to 10^9/ml. Aliquots of platelets (0.5 ml) were dispensed into aggregometer cuvettes and the platelets stirred continuously throughout experimentation at 900 rpm. Platelets were treated and activated as described for individual experiments and at predetermined times platelet activation was terminated by the addition of 0.2 v/v ice cold 20% perchloric acid. The samples were kept on ice for 20 minutes to allow protein precipitation. The proteins were then sedimented by centrifugation for 15 minutes at 2000 x g (4°C). The supernatants containing the water soluble Ins(1,4,5)P_3 were removed from each sample and titrated to pH 7.5 with 1.5 M KOH containing 60 mM HEPES buffer and universal indicator (Palmer *et al.*1986). The precipitated $KClO_4$ was then sedimented and removed as described above. Ins(1,4,5)P_3 standards (0.19 - 25 pmol/100 μl) were diluted into the same buffer as the samples (ie HBT to which 0.2 v/v ice cold 20% PCA had been added, which was then centrifuged, the supernatant titrated to pH 7.5 with 1.5 M KOH containing 60 mM HBT and universal indicator and which was then centrifuged again to remove precipitated $KClO_4$). Therefore standards and samples were in the same final media. To minimise loss of phosphorylated inositol species plasticware rather than glass was used throughout the extraction procedure.

2.2.10.2 Ins(1,4,5)P_3 assay

The samples were then assayed for Ins(1,4,5)P_3 using the D-myo-inositol(1,4,5)trisphosphate assay system using an adaptation of a previously described method (Challiss *et al.*1988). The assay is based on competition between unlabelled Ins(1,4,5)P_3 and a fixed quantity of [^{3}H]Ins(1,4,5)P_3 for a limited number of binding sites on a bovine adrenal protein preparation. The amount of [^{3}H]Ins(1,4,)P_3 binding is inversely proportional to the concentration of non-radiolabelled ligand. Bound [^{3}H]Ins(1,4,5)P_3 was separated from non-bound

[^{3}H]Ins(1,4,5)P_3 by centrifugation. The supernatant was decanted and any adherent drops of supernatant removed using a swab to avoid disturbing the pellet. The pellet was then resuspended, transferred to a scintillation vial and 4 mls of scintillant added. The bound [^{3}H]Ins(1,4,5)P_3 was counted in a ß scintillation counter and the amount of unlabelled Ins(1,4,5)P_3 per tube was determined by interpolation from a standard curve. A representative standard curve is given in figure 6.

In summary:-

[^{3}H]Ins(1,4,5)P_3 ↓ [^{3}H]Ins(1,4,5)P_3-bound

+ binding protein =

↑ Ins(1,4,5)P_3 Ins(1,4,5)P_3-bound

The sensitivity of the assay was <0.2 pmols, whilst the working range was 0.19 - 25 pmol/tube. The assay is specific for the (1,4,5)-isomer of IP_3 with the ability to cross react with Ins(1,3,4)P_3 500 times less than for Ins(1,4,5)P_3.

2.2.10.3 Recovery of Ins(1,4,5)P_3

The recovery of Ins(1,4,5)P_3 using the extraction system described in this assay was examined by extracting a known concentration of Ins(1,4,5)P_3 from a sample using the same method as employed to extract Ins(1,4,5)P_3 from a platelet sample. Using this method extraction of Ins(1,4,5)P_3 was consistently found to be greater than 85%.

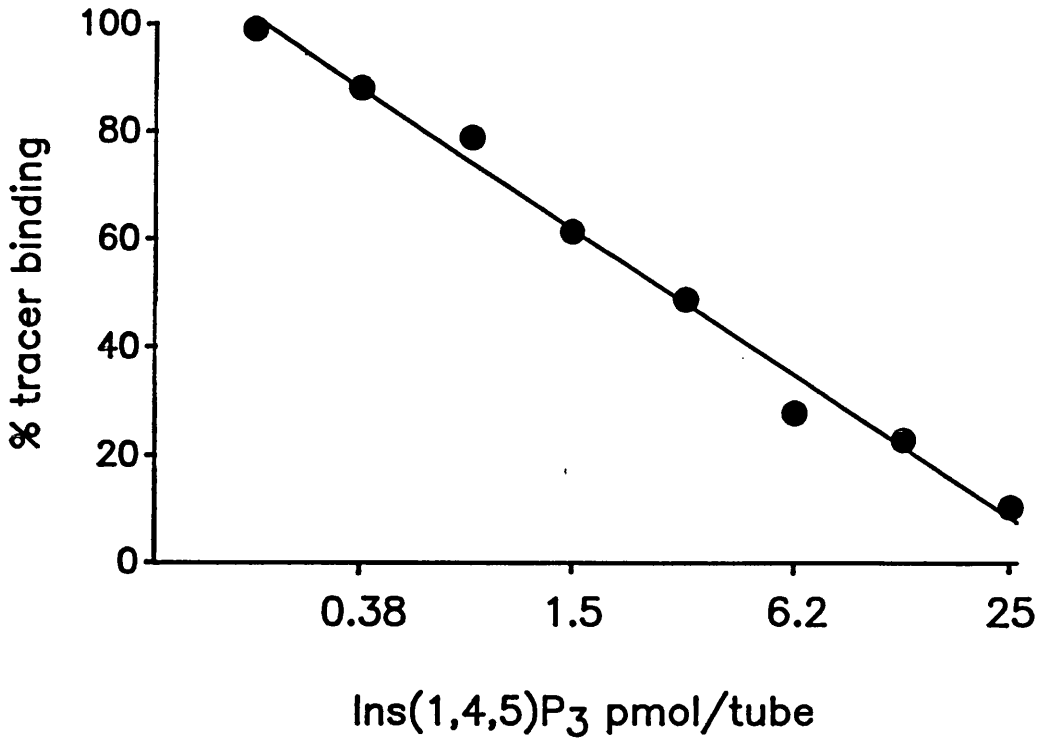

Figure 6 A standard curve for the measurement of Ins(1,4,5)P_3. The concentration of Ins(1,4,5)P_3 in the standards is inversely proportional to the binding of [^{3}H]Ins(1,4,5)P_3 to the specific binding sites for Ins(1,3,4)P_3 on the bovine adrenal protein preparation.

2.2.11 sn-1,2-DAG DETERMINATION

2.2.11.1 Sample preparation

Washed platelets were prepared as described in section 2.2.3 and washed twice before the final platelet count was adjusted to 10^9/ml. Aliquots of platelets (1 ml) were dispensed into aggregometer cuvettes and were continuously stirred at 900 rpm throughout experimentation. After the platelets had been treated (as described in individual experiments), activation was terminated and DAG extracted using a modification of the method of Bligh and Dyer (1959). At a pre-determined time a sub-aliquot of platelets (0.8 ml) was transferred into 3 mls of ice-cold chloroform:methanol 1:2 (v/v) and the monophase mixed thoroughly. Chloroform (1ml) and NaCl (1ml) were added to break phase and the samples were centrifuged at 2000 x g for 5 minutes. The lower chloroform phase was dried under nitrogen. The samples were then assayed for sn-1,2-DAG within 24 hours using the sn-1,2-diacylglycerol assay reagent system which is based on the method described by Preiss *etal* (1986).

2.2.11.2 Assay for DAG

The technique used was a radio-enzymic assay using *E.coli* DAG kinase and defined mixed micelle conditions to solubilize the DAG present (in either standards or samples) and allow its quantitative conversion to [^{32}P]phosphatidic acid in the presence of [^{32}P]-γ-ATP. In brief, the samples were resuspended in detergent solution reagent (supplied with the assay), sonicated and reaction mixture (containing DAG kinase and assay buffer) was added (also supplied). The reaction was initiated by adding *5 mM ATP containing 1 μCi [^{32}P]-γ-ATP to each tube and incubated at 25°C for 30 minutes. The reaction was terminated by adding perchloric acid and chloroform:methanol (1:2, v/v). The free [^{32}P]-ATP was then separated from the [^{32}P]-PA by washing and centrifugation finally leaving [^{32}P]-PA dissolved in chloroform.

*The concentration of ATP was crucial for the assay and therefore the concentration was confirmed using the absorbance of the ATP solution at 259 nm (where 15.4 x 10^3 is the molar extinction coefficient of ATP).

C18:0 C18:0

C20:4 DAG KINASE → C20:4
[^{32}P]Phosphatidic acid

C-OH O-[^{32}P]

DAG (1,stearoyl-2-arachidonyl) [^{32}P]-PA

After conversion of sn-1,2-DAG to [^{32}P]PA, standards which contained predominantly [^{32}P]PA (figure 7) were counted without need for further processing. The [^{32}P]PA contained in samples from platelet extracts however, contained other [^{32}P] labelled species (most prominently ceramide phosphate, known to be a substrate for *E.coli* DAG kinase (Preiss *et al.*1986)) which needed to be separated using thin layer chromatography (TLC). TLC plates were pre-activated in acetone and air dried. After addition of samples and authentic phosphatidic acid (both dissolved in chloroform:methanol (95:5 v/v)) the TLC plates were placed in paper lined tanks where the atmosphere was pre-saturated with the developing solvent (Chloroform:methanol:acetic acid/65:15:5 v/v/v). When the solvent reached the top of a TLC plate the plate was removed and air dried. The authentic PA was visualised by placing the TLC plates in a tank containing iodine and the area corresponding to phosphatidic acid marked.

2.2.11.3 Autoradiography

The TLC plates were subjected to autoradiography for 18 h at - 80°C. The autoradiograph film was developed and the areas on the TLC plates corresponding to [^{32}P]PA (RF value = 0.35) were cut from the TLC plate and placed into correspondingly labelled scintillation vials. After addition of 10 mls of scintillant the radioactivity attributable to [^{32}P]PA was determined by scintillation counting. The amount of DAG present in a sample was then calculated from the amount of [^{32}P]PA produced, taking into consideration the specific activity of the [^{32}P]-γ-ATP and after correcting for the proportion of the total volume of the sample which was taken for TLC separation.

The concentration of PA produced (pmol/tube) for each standard was calculated and plotted against the original concentration of DAG. The conversion of PA in each sample tube was then read for each sample directly from the standard curve (figure 7).

The assay is specific for biologically relevant sn-1,2-diacylglycerols as opposed to 1,3 -disteroyldiacylglycerol, however, it is possible that the assay may also be converting 1-alkyl,2-acyl glycerols and dialkylglycerols into [^{32}P]-Phosphatidic acid (Thompson *et al.*1990). The range of this assay is from 31 - 1000 pmols/tube and the sensitivity is 10 pmols/tube.

2.2.11.4 Improvements made to assay

1. Addition of nonradioactive PA to samples before running on TLC, allowed visualisation of PA using iodine without the need to undergo autoradiography.

2. A total count of [^{32}P]-*r*-ATP was taken, counting the exact amount added to each of the reactions (about 1 μCi) as this was more accurate than calculating the specific activity.

2.2.11.5 Recovery of DAG

The recovery of DAG was determined by extracting a known concentration of DAG from a sample using the same method employed for the extraction of DAG from a platelet sample. Extraction of DAG from a sample using the Bligh and Dyer method (Bligh and Dyer, 1959) as previously described followed by the conversion of DAG to [^{32}P]PA, and the extraction of this by thin layer chromatography gave a recovery of DAG which was consistently >90%.

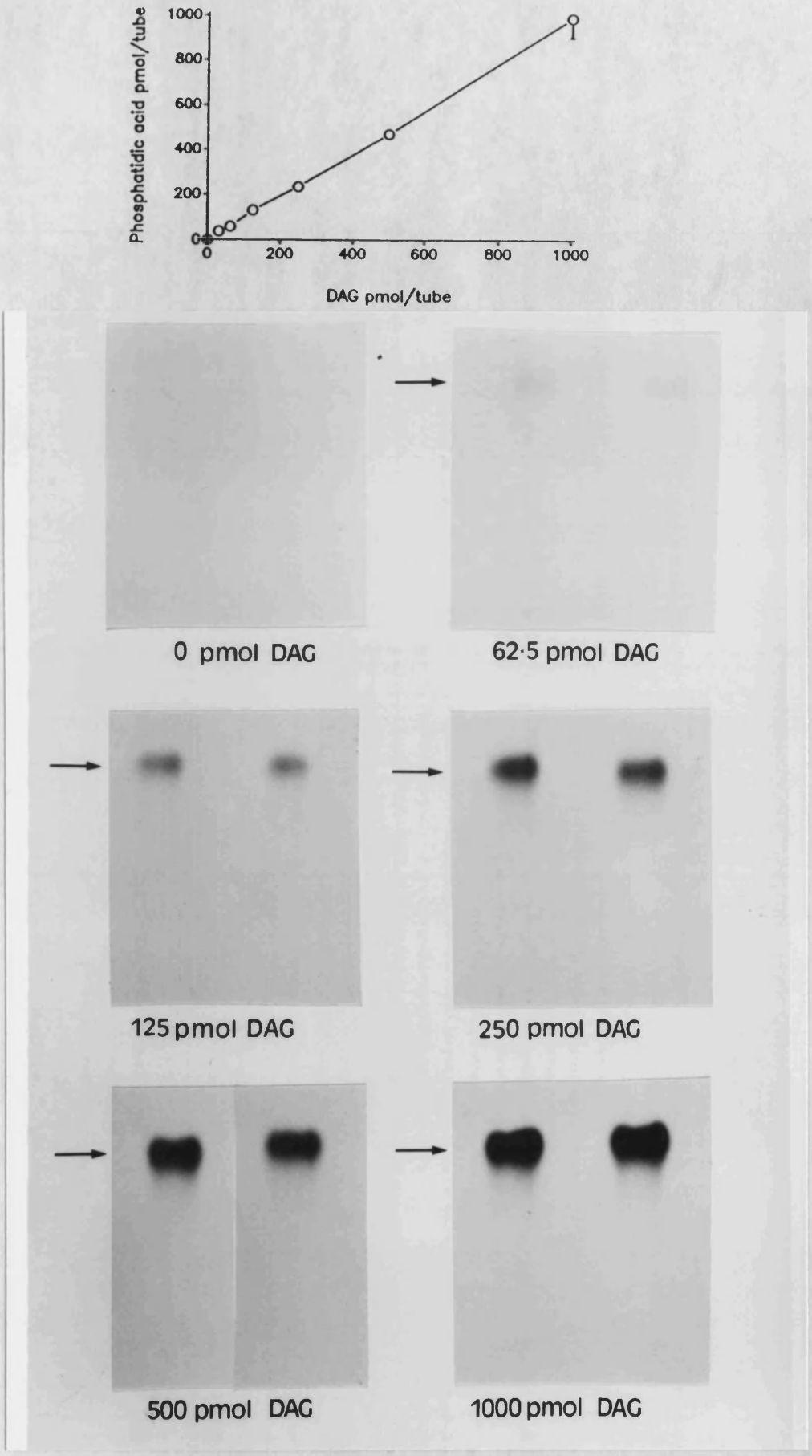

Figure 7 A standard curve for the conversion of sn-1,2-DAG to [^{32}P]PA in the presence of [^{32}P]ATP and DAG kinase. The phosphorylated PA shown in the autoradiographs was excised and subject to scintillation counting and the results given in the upper panel.

2.2.12 PROTEIN PHOSPHORYLATION AND ONE DIMENSIONAL GEL ELECTROPHORESIS OF PROTEINS

2.2.12.1 Sample Preparation

Sample Buffer

10% SDS	0.5 ml
glycerol	0.5 ml
1 M Tris pH 6.8	0.4 ml
Distilled H_2O	3.1 ml
2-mercaptoethanol	5% final

bromophenol blue added to attain desired depth of colour

Platelets were prepared as described in section 2.23 and the platelet pellet resuspended to 8 x 10^9 pl/ml in phosphate and BSA free HBT. The suspension was then incubated for 2 hours (rt) with 200 μCi [^{32}P]Pi/ml platelets. Excess [^{32}P]P_i was removed by washing the platelets 3 times in normal HBT and the final platelet count adjusted to 10^9/ml. Aliquots of platelets (300 μl, 10^9/ml) were dispensed into eppendorfs and treated as described in individual experiments. At a pre-determined time platelet activation was terminated and the protein in each sample solubilized by the addition of an equal volume of 2 x Laemmli SDS reducing gel-sample buffer (100°C) (Laemmli, 1970). The samples were then boiled for 5 minutes and centrifuged at 8000 x g to remove any particles.

2.2.12.2 SDS gel stock solutions

1. 30% acrylamide + 0.8% bisacrylamide, dissolved volumetrically in distilled water. Stored in dark at 0-4°C.
2. Running gel buffer: - 1 M Tris-HCl pH 8.8
3. Stacking gel buffer: - 1 M Tris-HCl pH 6.8
4. 10% SDS in distilled water
5. Molecular weight markers were dissolved in 1 x SDS sample buffer (37°C for 60 min) and then boiled for 5 minutes. Subaliquots were stored at -20°C.

Gel Preparation

	Running gel 10%		stacking gel 5%	
Acrylamide	20.0	(5)	6.68	(1.67)
Tris buffer	22.4	(5.6)	5.0	(1.25)
Water	17.4	(4.35)	24.0	(6.0)
10% SDS	0.6	(0.15)	0.4	(0.1)
10% ammonium persulphate	0.4	(0.1)	0.2	(0.05)
TEMED	0.04	(0.01)	0.04	(0.01)

The numbers given are the quantity of solutions required to run two 16 cm x 16 cm gels. The numbers given in brackets are the quantity of solutions required to run 2 of the mini-gels (see later).

Running Buffer

25 mM Tris base (3.03g/L)

192 mM glycine (14.42g/L)

0.1% SDS (1g/L)

Coomassie Blue Stain

40% methanol

53% distilled H_2O

7% acetic acid

0.1% (w/v) Coomassie Brilliant blue R

Destaining solution

40% methanol

53% distilled H_2O

7% acetic acid

In this study one dimensional gel electrophoresis was used which under denaturing conditions (in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode (Laemmli, 1970). After solubilizing all the proteins in a sample by boiling in the presence of SDS and 2-mercaptoethanol (which reduces disulphide bonds) an aliquot of the protein sample was applied to a gel lane and the individual proteins were separated electrophoretically using a Biorad Protean II system.

2.2.12.3 Gel preparation and one dimensional SDS gel electrophoresis

Polyacrylamide SDS gels (16 cm x 16 cm x 1mm) were poured using a 10% polyacrylamide SDS gel as the running gel and a 5% polyacrylamide SDS gel as the stacking gel. After pouring the running gel a 50% solution of methanol was layered onto it to prevent the formation of a meniscus and also to prevent oxidisation of the gel, the methanol was removed before the stacking gel was poured. Standard molecular weight markers were prepared as described in materials and 100 μl loaded into one of the tracks, whilst samples (containing 100 μg protein) were loaded into every other available track. The power supply was then connected to the cell (Biorad Protean IIxi system) and run at 160 volts until the bromophenol blue tracking dye entered the separating gel, the voltage was then increased to 300 volts. When the tracking dye reached the bottom of the separating gel, the power was disconnected and the gel removed and placed into Coomassie brilliant blue stain.

The sensitivity of Coomaissie blue staining is 0.3 - 1 μg protein/band. Coomaissie blue stain binds non-specifically to protein but not to the acrylamide gel. The gels were immersed in Coomaissie blue stain for 4 hours and gently agitated. The stain was then decanted off and the gels were immersed in destain solution. The destain solution was changed regularly until the blue bands of protein on a clear background were visible on the gels.

After staining, the gels were sandwiched between a sheet of Whatman 3MM filter paper (backing) and a sheet of clear cellophane (front) and dried at 80°C for 80 minutes using a gel dryer (Biorad, model 853). When dry the gels were subject to autoradiography in order to detect the [^{32}P]P_i containing protein bands. The autoradiograph film was sandwiched between the dried gel and an intensifying screen, and the corners of the gel were marked on the film. The gel, film and intensifier were then positioned into a light sealed autoradiography cassette and placed at -80°C for 18 hours. The autoradiograph film was then developed until

the required intensity of darkness was obtained and after washing was then fixed for 2 minutes. The film was then washed and the area on the gel corresponding the phosphorylated PKC substrate was excised and placed in a correspondingly labelled scintillation vial. Scintillant (10 mls) was then added to each vial and the radioactivity from each sample was assessed by scintillation spectroscopy in a ß-scintillation counter.

2.2.12.4 Percentage gels

Depending on whether it is high molecular weight proteins which are of interest or low molecular weight proteins, by varying the percentage of acrylamide in the gel, the proteins will either move more quickly through the open matrix of a lower percentage acrylamide gel or more slowly through the closer matrix of a higher percentage acrylamide gel. The higher the molecular weight of the protein of interest therefore, the lower the percentage of acrylamide in the gel which is required in order for that protein to be separated from others of a similar molecular weight on the gel.

2.2.13 WESTERN BLOTTING

2.2.13.1 Antibodies

Primary antibodies

Anti-phosphotyrosine (PY20) (Glenney *et al.*1988) monoclonal antibody from mouse hybridomas (1 mg/ml in PBS) was purchased from ICN Biochemicals and stored at 4°C. It was used at a 1:500 dilution.

Anti-phosphotyrosine (06-123) polyclonal antibody produced in rabbit (1 mg/ml in PBS) was purchased from Upstate Biotechnology Inc. Lake Placid, New York. The antibody was sub-aliquoted, stored at -20°C and used at a 1:500 dilution.

Anti-annexin I antibody (code 842), anti-annexin IV antibody (code 179) and anti-annexin V antibody (code 890) all produced in rabbit were gifts from Professor R. Flower, The William Harvey Institute, St.Bartholomews Hospital, London. The antibodies were stored at -20°C and used at a dilution of 1:5000.

Anti-annexin I (against amino acid sequences 1-188 [code I], 24-37 [code 13] and 38-50 [code 31]) and anti-annexin II [code II] antibodies produced in rabbit were gifts from Dr. F. Carey, ICI, Macclesfield, U.K. They were sub-aliquoted, stored at -20°C and used at a dilution of 1:500.

Anti-vinculin antibody produced in mouse was a gift from Dr. M. Wilkinson, Royal College of Surgeons. It was used at a 1:1000 dilution and stored at 4°C.

Second antibodies

Goat Anti-Mouse IgG (H + L) horseradish peroxidase conjugate and Goat Anti-Rabbit IgG (H + L) horseradish peroxidase conjugate were purchased from Bio-Rad Laboratories, stored at 4°C used at a 1:500 dilution.

Peroxidase anti-peroxidase antibodies

Peroxidase anti-peroxidase complex antibody (rabbit) and Peroxidase anti-peroxidase complex antibody (mouse) were purchased from Sigma, stored at 4°C and at -20°C respectively, and used at a 1:10,000 dilution.

2.2.13.2 Western Blotting - Theory

Western blotting allows the identification of a particular protein by using the specificity inherent in antigen:antibody recognition. Using this method < 1 ng of antigen can be detected. In order to detect an antigen by Western blotting the proteins in a sample first have to be separated using one dimensional gel electrophoresis and then transferred from the gel to nitrocellulose transfer membrane. The non-specific binding sites on the nitrocellulose are then blocked to prevent non-specific binding of antibody and then probed with the antibody of interest. The antibody is then detected by a horseradish peroxidase (HRPO) anti-immunoglobulin (Ig) conjugate and visualised by incubating the nitrocellulose in the presence of 3,3'diaminobenzidine. Before starting the Western blotting technique it was necessary to optimise the conditions in order to achieve the best sensitivity, but to avoid non-specific binding of antibodies.

2.3.13.3 Optimising Conditions for Western blotting

(a) Determination of an optimal time for transfer of proteins from the polyacrylamide SDS gel to the nitrocellulose membrane. In order to determine an optimal time for transfer of proteins, different lengths of time were tested for the transfer. At predetermined times the amount of protein remaining in the polyacrylamide SDS gel was detected using Coomassie blue stain, the protein transferred to the nitrocellulose was detected using Ponceau S staining, and any protein which had passed through the nitrocellulose, was detected by placing a second sheet of nitrocellulose behind the first when transferring the proteins, and then detecting protein by staining with Ponceau S. Using this system, the optimum time for the transfer of protein was found to be 2 hours 30 minutes using a constant current of amps equal to the number of cm^2 of the gels to be transferred. At this time point, only the very highest molecular weight proteins remained on the polyacrylamide SDS gel, and no protein had passed through to reach the second sheet of nitrocellulose. Ponceau S staining showed that most of the protein was transferred to the nitrocellulose sheet in direct contact with the gel. As a control the SDS polyacrylamide gel was routinely stained with Coomaissie Blue after the transfer of protein to the nitrocellulose in order to ensure adequate transfer of protein had taken place.

(b) Determining the best blocking solution to use to prevent binding of antibodies to the non-specific binding sites on the nitrocellulose. From the literature the most commonly used methods for blocking non-specific binding include the use of gelatin, BSA, non-ionic detergents and milk powder, or a combination of these (Stott, 1989). Experiments were performed to determine which of these methods was the best to prevent non-specific binding in the system being used in this study. Several permutations were assessed, using each of these agents alone and in combination with each other. Of the blocking solutions tried, the one which was best at preventing non-specific binding in this system used was 3% gelatin dissolved in 170 mM NaCl + 10 mM Tris (pH7.4).

(c) Determining the optimal time and conditions for the incubation of the antibodies. It was found that if the nitrocellulose was incubated overnight with the first antibody at room temperature, detection of the antigen was improved over an incubation period of one hour at room temperature. However, it was found that if the nitrocellulose was gently agitated during incubation of the primary antibody, non-specific binding to the nitrocellulose increased. Increasing the incubation time of the second antibody to greater than one hour 30 minutes at room temperature did not improve detection of the antigen. To determine the concentration of the primary antibody to use for Western blotting, dilutions of the antibody were made as recommended from their source, and then changed if necessary to improve detection or prevent non-specific binding.

(d) Ascertaining the best solution for washing the nitrocellulose. Washing of the nitrocellulose membranes between antibody incubations was important to prevent non-specific binding. Several methods were tried to determine optimum washing conditions. These methods included:-

a. Phosphate buffered saline (+ 0.1% Tween-20)
b. 170 mM NaCl + 10 mM Tris (pH 7.4) (+ 0.05% Tween-20)
c. 30 mM NaCl + 10 mM Tris (pH 7.4) + (0.1% Tween-20)

The method which gave the cleanest results was 170 mM NaCl + 10 mM Tris (pH 7.4) (+ 0.05% Tween-20).

(e) Enhancement of the antigen signal. Several techniques are available which will enable enhancement of the antigen signal on Western blotting, including the use of peroxidase anti-peroxidase antibody (Frazer and Wisdom, 1985) and nickel and cobalt enhancement (Stott, 1989). The use of peroxidase anti-peroxidase antibody was found to significantly enhance the antigen signal when used at a very low concentration (1 in 10,000) [see figure 8]. However, using nickel and cobalt increased the colour of the background and therefore reduced the differential between signal and background.

(c) Determining the optimal time and conditions for the incubation of the antibodies. It was found that if the nitrocellulose was incubated overnight with the first antibody at room temperature, detection of the antigen was improved over an incubation period of one hour at room temperature. However, it was found that if the nitrocellulose was gently agitated during incubation of the primary antibody, non-specific binding to the nitrocellulose increased. Increasing the incubation time of the second antibody to greater than one hour 30 minutes at room temperature did not improve detection of the antigen. To determine the concentration of the primary antibody to use for Western blotting, dilutions of the antibody were made as recommended from their source, and then changed if necessary to improve detection or prevent non-specific binding.

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The method given below for Western blotting is a combination of the optimal conditions found for transferring protein and blocking, incubating and washing the nitrocellulose.

2.2.13.4 Stock solutions

Tris buffered saline
170 mM NaCl
10 mM Tris (pH 7.4)

Blocking Buffer
3% gelatin in Tris buffered saline

Blotting Buffer
50 mM Tris-Base
40 mM glycine
0.02% SDS
20% methanol

Ponceau S stain
0.1% Ponceau S
1% aqueous acetic acid

Ponceau S destain
200 μM NaOH

3,3'diaminobenzidine substrate solution
50 mg DAB in 200 mls PBS (filtered)
100 μl H_2O_2 added directly before use.

2.2.13.5 Preparation of samples for Western blotting

Western blotting was used for probing washed human platelet lysates for annexins (I,II,IV and V) and for probing washed rabbit platelet lysates for phosphotyrosine. Human platelets were isolated and prepared as described in section 2.2.3.4 and rabbit platelets were isolated and prepared as described in section 2.2.3.

2.2.13.5.1 Preparation of human platelet samples to probe for annexins

Total platelet protein was obtained by resuspending human platelets to a concentration of 5 x 10^9/ml and adding an equal volume of 2 x Laemmli SDS reducing gel-sample buffer (100°C) (Laemmli, 1970). In order to detect EDTA/EGTA releasable annexin from the outer surface of the plasma membrane, 5 x 10^9 platelets were washed in 5 mM EGTA/5 mM EDTA and an equal volume of 2 x Laemmli SDS reducing gel-sample buffer (100°C) added to the supernatant. The remaining platelet pellet was resuspended to its original volume in 1 x Laemmli SDS reducing gel-sample buffer (100°C). In order to assess for the release of annexins from the platelet granules, platelets were stimulated with various agents in the presence of 1 mM $[Ca^{++}]_i$ whilst being continually stirred (900 rpm). The reaction was terminated at 3 minutes after activation by removing a sub-aliquot of platelets into 5 mM EGTA/5 mM EDTA centrifuging and then adding an equal volume of 2 x Laemmli SDS reducing gel-sample buffer (100°C) to the supernatant. The remaining pellet was resuspended to its original volume in 1 x Laemmli SDS reducing gel-sample buffer (100°C). All samples were boiled for 5 minutes and then centrifuged (12,000 x g, 2 minutes) before the supernatants were run on polyacrylamide SDS gels.

2.2.13.5.2 Preparation of rabbit platelet samples to probe for phosphotyrosine

Washed rabbit platelets were resuspended to 1-2 x 10^9 platelets/ml and all experiments were carried out at 37°C. Platelets were continuously stirred (900 rpm) throughout experimentation. To terminate the reactions an equal volume of 2 x Laemmli SDS reducing gel sample buffer (100°C) was added and the samples were boiled for 5 minutes and centrifuged (12,000 x g, 2 minutes) before the supernatants were run on polyacrylamide SDS gels.

2.2.13.5.3 Treatment of U937 cells with TPA

U937 cells were grown in 10 % fetal calf serum and 90 % RPM-I in the presence of low concentrations of fungizone (5 μg/ml), penicillin (10 μg/ml) and streptinomycin (10 units/ml). When they reached a concentration of 10^6 cells/ml they were transformed by the addition of 50 ng TPA/ml for 24 hours. The transformed U937 cells were used as a positive control for the presence of annexins on Western blots (Isacke *et al.*1989). After transformation the cells were washed in HBT containing 1 mM $CaCl_2$ and they were then resuspended to 5 x 10^6 cells/ml in Laemmli SDS reducing gel-sample buffer, boiled for 5 minutes and centrifuged (12,000 x g) for 5 minutes before being run on the Western blots. I would like to acknowledge Mr R. Pleass for growing the U937 cells.

2.2.13.6 One dimensional gel electrophoresis of proteins

Mini-gels (8 cm x 8 cm x 0.75 mm) were poured using the volume of reagents described in section 2.2.12.5 (the figure in brackets is sufficient for 2 minigels). The percentage of the polyacrylamide SDS gel used as the separating gel varied depending on the individual experiments and is given in the figure legends. However the stacking gel was always a 5% polyacrylamide SDS gel. Protein samples of interest were solubilized by adding an equal volume of 2 x Laemmli SDS reducing gel sample buffer (100°C) and boiled for 5 minutes (Laemmli, 1970). Molecular weight markers (10μl) were added to one track, whilst the samples (containing about 10 μg protein/track) were dispensed into the remaining tracks. The power supply was then connected to the cell (Biorad miniprotean II™) and the gels were run at 150 volts until the bromophenol blue tracking dye reached the bottom of the separating gel. The power was then disconnected and the gels placed in blotting buffer for 15 minutes.

2.2.13.7 Transferring Proteins from gels to nitrocellulose

Proteins were transferred from gel to nitrocellulose membrane (Towbin *et al.*1979) using a Semi-Dry Blotter (Sartorius, Sartoblot II-S). Twelve sheets of Whatman 3 MM filter paper and one piece of nitrocellulose were cut to the same size as the gel and were soaked in blotting buffer. The filter paper (6 pieces) was placed on the cathode side of the semi-dry blotter, followed by the gel, then the nitrocellulose and finally by another six pieces of the filter paper. Any air bubbles in the 'sandwich' were expelled and the anode side 'lid' of the semi-dry blotter was closed. The direction of transfer is from the cathode side to the anode side and therefore protein was transferred from the gel onto the nitrocellulose.

The number of mAMPs used to transfer protein depended on the surface area of the total number of gels to be transferred with 1 cm^2 of gel requiring 1 mAMP for 2.5 hours. Therefore 2 gels of 6 x 8 cm equalling a total surface area of 96 cm^2 were transferred at 96 mAMPs for 2.5 hours. After removing them from the semi-dry blotter, the gels were stained using Coomaissie Blue stain in order to assess protein transfer whilst the nitrocellulose membranes were (as required) stained in Ponceau S in order to check transfer or to enable cutting them into tracks. The nitrocellulose was then destained for 10 minutes and placed in blocking solution for a further 1.5 hours in order to block non-specific antibody binding sites on the nitrocellulose.

2.2.13.8 Western Blotting

After blocking the non-specific binding sites the gels were washed twice in Tris-buffer (170 mM NaCl, 10 mM Tris-HCl [pH 7.4]) for 10 minutes each, followed by two washes in Tris-buffer plus 0.05% tween-20 (TBT-20). For each of the nitrocellulose membranes a square petri dish lined with parafilm was prepared, the nitrocellulose was placed on the parafilm (which repels any moisture) and the primary antibody (diluted in TBT-20) was gently pipetted onto the membrane. The lid was then placed on the dish and the primary antibody incubated overnight at room temperature.

The non-specifically bound primary antibody was removed by washing the nitrocellulose three times in TBT-20. The second antibody (horseradish peroxidase anti-IgG conjugate) was diluted in TBT-20, added to the gels (5 mls/gel) and incubated for 1 h at rt. Non-specifically bound second antibody was washed away by rinsing the membrane three times (10 minutes each) in TBT-20, a 1:10000 dilution of peroxidase-anti-peroxidase antibody was then added to the gels and incubated for 1 hour. This was washed away with TBT-20 followed by washing twice in Tris-buffer (10 minutes each) and finally rinsing briefly in PBS. DAB substrate solution (50 mls per gel) was added to the nitrocellulose and when the colour had developed it was transferred to distilled H_2O to stop the reaction. Figure 8 demonstrates the enhancement of the signal which is achieved by the addition of the peroxidase anti-peroxidase antibody compared to developing the Western blot without using peroxidase anti-peroxidase enhancement.

2.2.13.9 Relative Optical Densitometry

The relative optical density of tyrosine phosphorylated protein bands on the Western blots was determined using a Joyce-Loebl Chromoscan-3 with a 0.1 x 3 mm aperture setting.

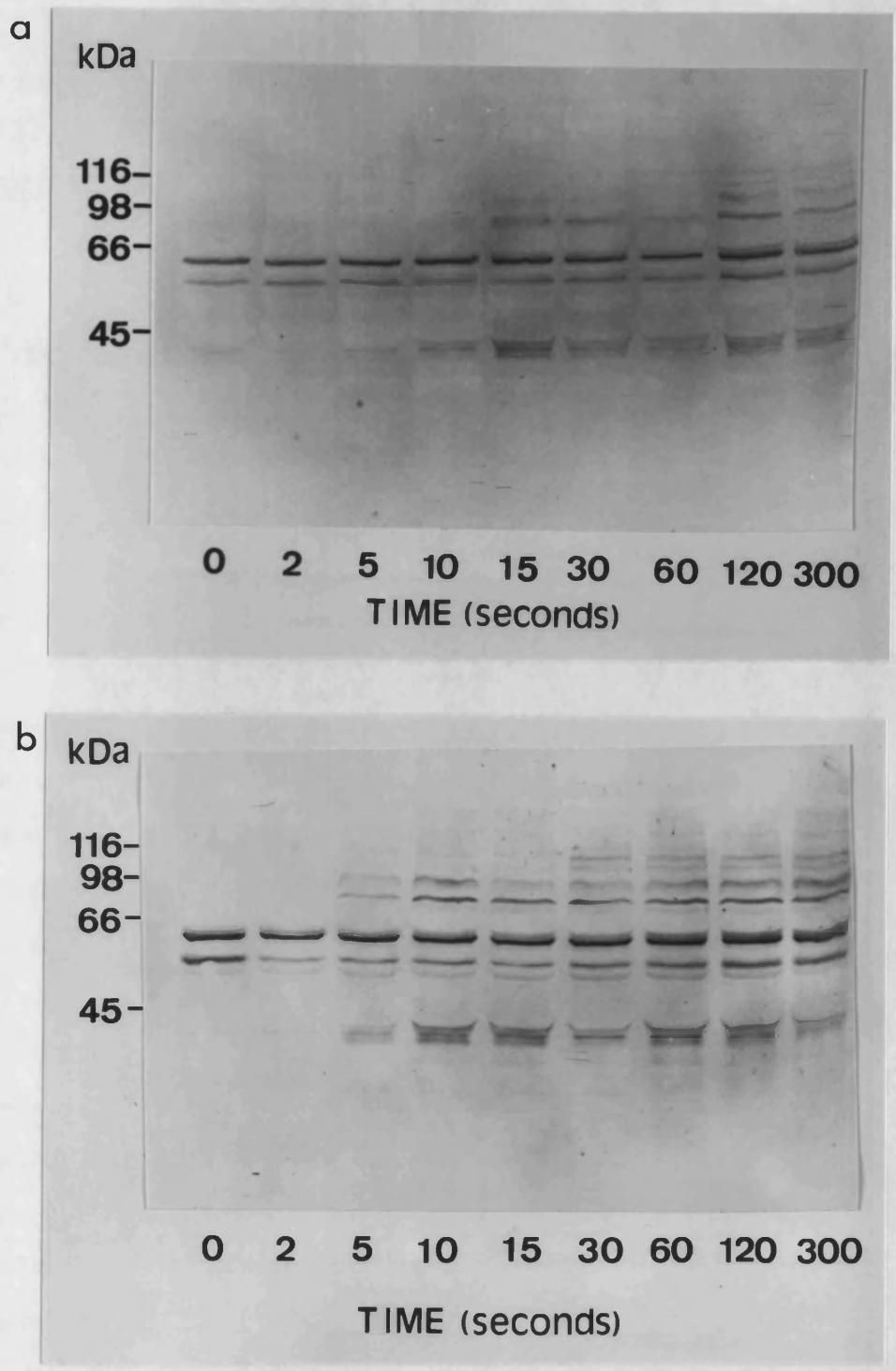


Figure 8 To demonstrate the enhancement of the signal for the proteins detected by Western blotting by using a peroxidase anti-peroxidase antibody. (a) A time course of tyrosine phosphorylation of platelet proteins induced by 300 nM PAF in the absence of peroxidase anti-peroxidase antibody (b) the same time course as in (a) using a 1:10,000 dilution of peroxidase anti-peroxidase antibody as a third antibody to enhance the signal.

2.2.14 ELISA FOR ANNEXIN 1

The enzyme-linked immunosorbent assay (ELISA) for annexin 1 (Smith *et al.* 1990) was carried out, on samples prepared by myself, by Dr S. Peers at the William Harvey Institute, St. Bartholomews Hospital, London.

2.2.14.1 Preparation of samples for ELISA

Washed human platelets were prepared as described in section 2.2.3.5 and resuspended to a final concentration of 5 x 10^9/ml. In order to determine the total annexin I concentration in the platelets, they were suspended in HBT containing 5 mM EGTA/5mM EDTA and subjected to rapid freeze/thawing at least five times before centrifugation (12,000 x g, 2 mins). The supernatant was then assessed for annexin I.

In order to release cell surface annexin, cells were treated with 5 mM EGTA/5 mM EDTA and then centrifuged (12,000 x g, 2 mins) to remove the supernatant which was assessed for annexin I. The remaining platelet pellet was subjected to freeze/thawing at least 5 times and then centrifuged (12,000 x g, 2 minutes) and the supernatant assessed for annexin I.

In order to examine for release of annexin I from the dense granules, platelets were stimulated with various agonists, whilst being continually stirred (37°C) in the presence of 1 mM $[Ca^{++}]_e$. The reactions were terminated at three minutes after platelet activation by removing a subaliquot into ice-cold tubes containing 5 mM EDTA/5mM EGTA. The samples were centrifuged as described above and the supernatant assessed for annexin I. In order to determine how much granule release had occurred, a parallel experiment was performed using ^{14}C-5HT loaded platelets as described in section 2.2.8.1 which were treated in an identical way to those to be assessed for release of annexin I. From this it was possible to determine the percentage ^{14}C-5HT for each individual treatment.

2.2.14.2 ELISA assay for annexin I

The ELISA assay for annexin I (Smith *et al.*1990) has a sensitivity range of 0.1 - 2000 ng/ml. The ELISA used was a sandwich assay with a monoclonal anti-annexin I antibody (code 1A) used to coat the plate, the samples were added and any annexin I contained in them was bound to the plate, a polyclonal anti-annexin I antibody (code 842) was then added to recognise the bound annexin I. This two antibody method is thought to be specific for only annexin I and its degradation products.

2.2.15 LACTATE DEHYDROGENASE

Leakage of the enzyme lactate dehydrogenase from cells is an indicator of cell damage and increased permeability. In this study lactate dehydrogenase activity has been assessed from the supernatants of platelets which have been stimulated with AA or ionomycin.

2.2.15.1 Preparation of Samples

Platelets were prepared as described in sections 2.2.3 and were resuspended to a final concentration of 5 x 10^8/ml. 1 ml aliquots were dispensed into eppendorfs, $[Ca^{++}]_e$ adjusted to 1 mM and the platelets allowed to equilibrate to 37°C before activation. The platelets were then activated with a dose range of ionomycin or AA and mixed. The platelets were centrifuged at 12,000 x g for 2 minutes and the supernatant removed and placed on ice until lactate dehydrogenase assessment. As a positive control platelets were lysed with 1% triton-X-100 and the supernatant removed as described above. Release of lactate dehydrogenase from untreated platelets was determined in order to assess basal levels of lactate dehydrogenase.

2.2.15.2 Lactate Dehydrogenase Assay

Lactate dehydrogenase activity was determined using a kit from Sigma which is based on the spectrophotometric method of Wroblewski and LaDue (1955). This assay measured the rate at which the substrate pyruvate was reduced to lactate by lactate dehydrogenase.

Pyruvate + NADH ← Lactate dehydrogenase → Lactate

[High A_{340}] + NAD

[low A_{340}]

The reaction is coupled with the oxidation of nicotinamide adenine dinucleotide reduced form [NADH] which is followed spectrophotometrically in terms of reduced absorbance at 340 nm every 30 s for 3 minutes. The change in absorbance was measured at a linear part of curve.

The activity in a sample was calculated using the formula:-

Serum LD activity (u/ml) = change absorbance/minute x 20000 x TCF

TCF = 1 at 25°C

2.2.16 STATISTICS

The number of times each experiment was performed is given in the respective figure legends and where possible the data is given as the mean result $\pm$ S.E.M of the total number of experiments. To determine if there were significant differences between the sets of data an Anova test was performed. If significant differences were found in the data, a paired Student's T test was employed to find which treatments caused these significant differences. The levels of significance are denoted by * = $P<0.05$, ** = $P<0.01$ and *** = <0.005. The Bonferroni correction was applied where necessary, ie. when comparing several sets of data to a single set of control data (Wallenstein *et al.* 1980).

3. RESULTS

3.1 Investigation of the role of PKC in PAF-stimulated functional responses

3.1.1 Characterisation of PAF, ionomycin and arachidonic acid-induced platelet functional responses

In order to investigate the effect of PKC activation or inhibition on either PAF, ionomycin or AA induced platelet functional responses it was important to determine a concentration of each of these platelet activators which would elicit a sub-maximal response for each of the functional assays in which it was to be used. The effects of increasing concentrations of PAF, ionomycin and AA on $[Ca^{++}]_i$ elevation and TxB_2 generation are shown in Figs 9, 10 and 11 respectively, and the effect of increasing concentrations of PAF and ionomycin on 5-HT release is shown in Fig 12.

Increasing concentrations of each of the three platelet stimulants PAF, ionomycin or AA caused a dose dependent increase in $[Ca^{++}]_i$ elevation, TxB_2 generation and 5-HT release. A concentration of 3 nM PAF caused a submaximal elevation of $[Ca^{++}]_i$ and a submaximal release of 5-HT (Fig 9 & 12). This concentration of PAF however, only caused trace amounts of TxB_2 generation above the basal level of 0.1 pmol/10^8 platelets (Fig 9), therefore a suboptimal concentration of 30 nM PAF was used for the TxB_2 experiments. A concentration of 5 μM ionomycin was found to be suboptimal for all three functional responses, $[Ca^{++}]_i$ elevation, TxB_2 generation and 5-HT release (Fig 10, 12). A concentration of 30 μM AA was sub-optimal for both $[Ca^{++}]_i$ elevation and TxB_2 generation (Fig 11). Pre-incubation of platelets with 2.8 μM of the cyclo-oxygenase inhibitor indomethacin caused total inhibition of TxB_2 generation in platelets stimulated with 30 nM PAF, 5 μM ionomycin or 30 μM AA (Figs 9, 10 & 11).

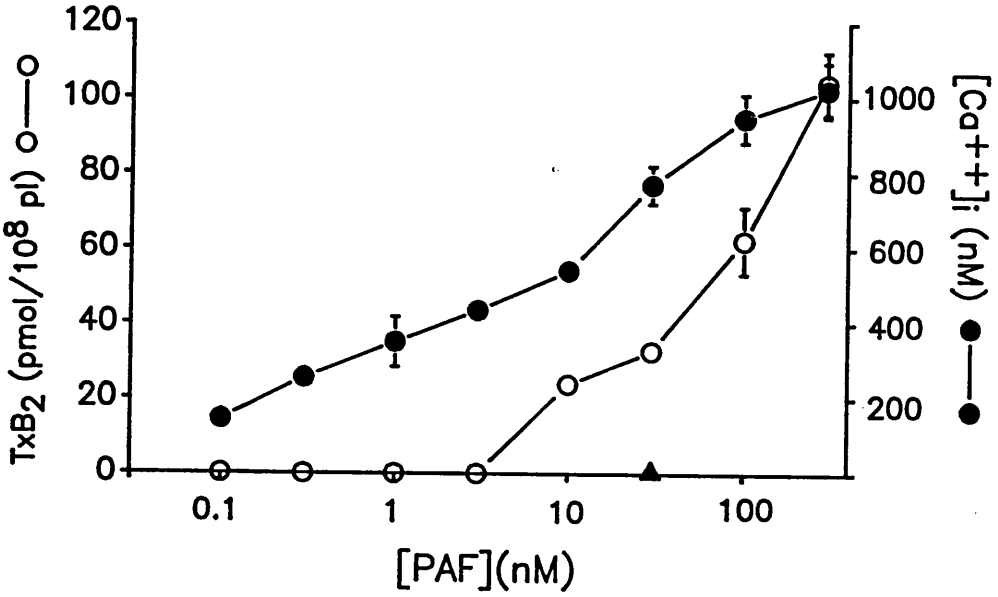


Fig 9 The effect of increasing concentrations of PAF on $[Ca^{++}]_i$ elevation and TxB_2 generation. Increasing concentrations of PAF were added to Fura-2 loaded platelets in the presence of 1 mM $[Ca^{++}]_e$. $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-PAF value from the peak value for $[Ca^{++}]_i$ after platelet activation. TxB_2 generation was determined at 3 minutes post-stimulation. Also shown as the closed triangle is the effect of 2.8 μM indomethacin on 30 nM PAF-induced TxB_2 generation. Each value represents the mean $\pm$ S.E.M. of at least three experiments each performed in triplicate.

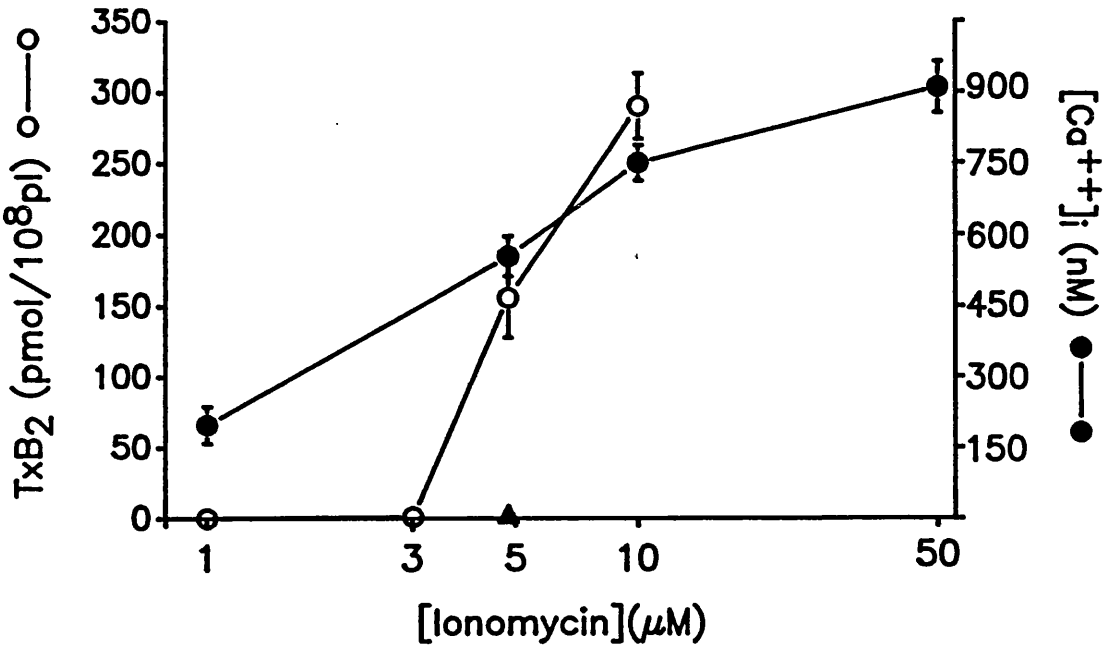


Fig 10 The effect of increasing concentrations of ionomycin on $[Ca^{++}]_i$ elevation and TxB_2 generation. Increasing concentrations of ionomycin were added to fura-2 loaded platelets in the presence of 1 mM $[Ca^{++}]_e$. $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-ionomycin value from the peak value for $[Ca^{++}]_i$ after platelet activation. TxB_2 generation was determined at 3 minutes post-stimulation. Also shown as the closed triangle is the effect of 2.8 μM indomethacin on 5μM ionomycin-induced TxB_2 generation. Each value represents the mean $\pm$ S.E.M. of at least three experiments each performed in triplicate.

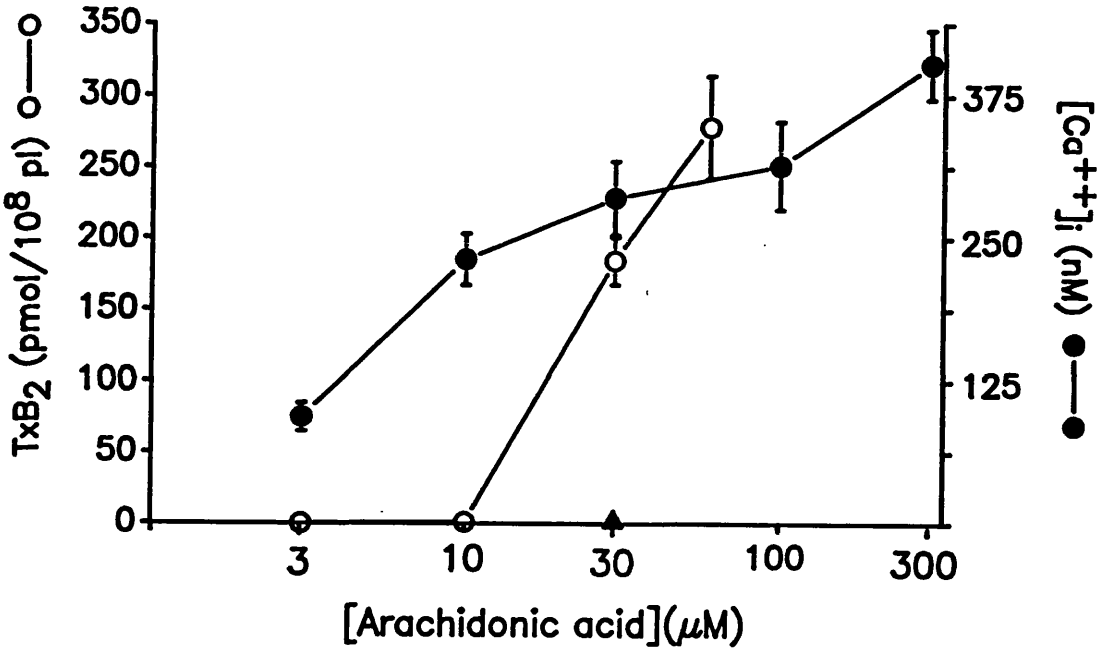


Fig 11 The effect of increasing concentrations of arachidonic acid on $[Ca^{++}]_i$ elevation and TxB_2 generation. Increasing concentrations of arachidonic acid were added to fura-2 loaded platelets in the presence of 1 mM $[Ca^{++}]_e$. $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-arachidonic acid value from the peak value for $[Ca^{++}]_i$ after platelet activation. TxB_2 generation was determined at 3 minutes post-stimulation. Also shown as the closed triangle is the effect of 2.8 μM indomethacin on 30 μM arachidonic acid-induced TxB_2 generation. Each value represents the mean $\pm$ S.E.M. of at least three experiments each performed in triplicate.

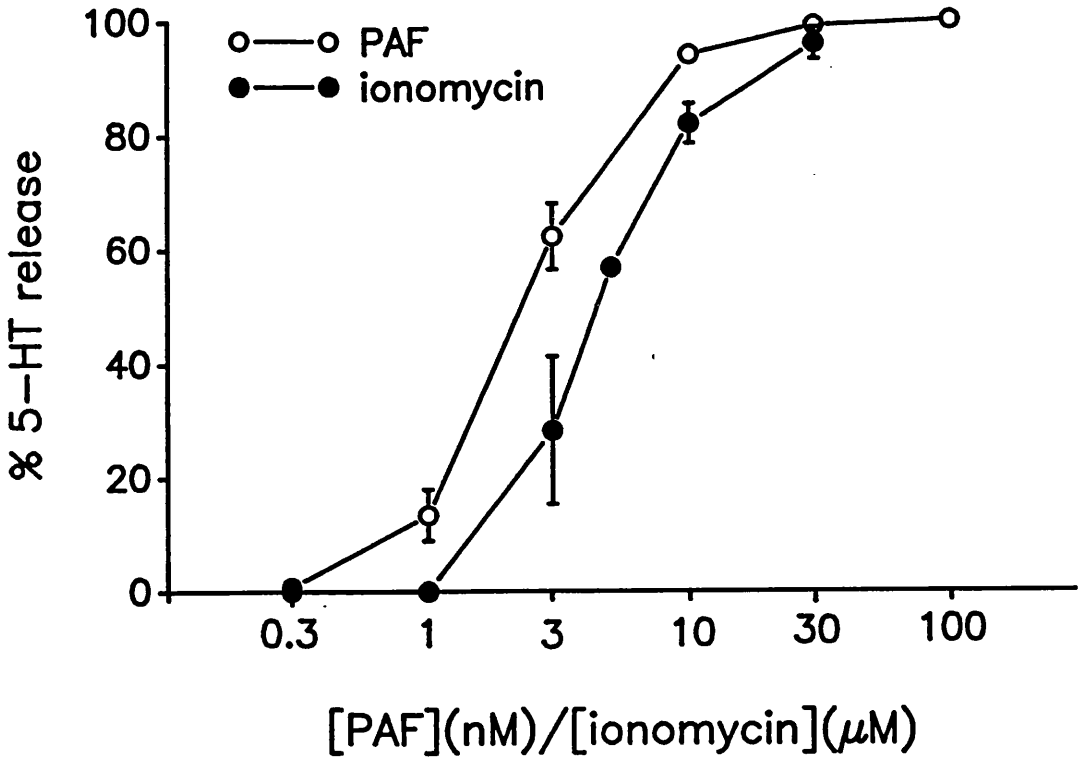


Fig 12 The effect of increasing concentrations of PAF or ionomycin on platelet dense granule release. 5-HT release was determined at three minutes post-stimulation. The points are the mean ± S.E.M of three experiments performed in triplicate.

3.1.2 Inhibition of PKC substrate phosphorylation by staurosporine

To determine whether staurosporine inhibits PKC activity and to find a suitable dose range of staurosporine to use in order to investigate the effect of PKC inhibition on platelet functional responses, the effect of a range of concentrations of staurosporine was examined on the phosphorylation of the major 40-47 kDa substrate of PKC. Addition of 300 nM PAF caused a 250% increase in phosphorylation of the PKC substrate (40-47 kDa) above basal levels (Fig 13, lanes 1 + 2). Increasing concentrations of staurosporine caused inhibition of phosphorylation of the PKC substrate in a dose dependent manner (Fig 13, lanes 3-6) over the range 30-1000 nM staurosporine with an IC_{50} of 500 nM.

3.1.3 PKC modulation and PAF-induced platelet activation

Activation of PKC in platelets with a 2 minute pretreatment using either 1-30 nM of 12-0-tetradecanyl phorbol-13-acetate (TPA) or 6.8-68 μM of the membrane permeant diacylglycerol sn-1,2-dihexanyl-sn-diacylglycerol (sn-1,2-DHG) caused a dose dependent inhibition of both the rate and maximal height of $[Ca^{++}]_i$ elevation induced by 3 nM PAF (Fig 14). In contrast 30-1000 nM staurosporine had no significant effect on either the rate or maximal height of 3 nM PAF-induced $[Ca^{++}]_i$ elevation (Fig 14). Neither TPA, DHG nor staurosporine had any effect on basal levels of $[Ca^{++}]_i$ which remained at 152 $\pm$ 11 (n=30). Concentrations of the non-esterified precursor molecule of TPA, namely 4ß-phorbol at concentrations of 1000 fold greater than those found to be active for TPA, had no effect on PAF-induced $[Ca^{++}]_i$ elevation (Fig 15). Similar to the results for $[Ca^{++}]_i$ elevation, stimulation of PKC with TPA caused a dose dependent inhibition of TxB_2 generation (Fig 16), whilst conversely, inhibition of PKC significantly potentiated TxB_2 generation but only at the highest concentration of 1 μM staurosporine in platelets stimulated with 30 nM PAF (Fig 16). Neither 30 nM TPA nor 1 μM staurosporine alone increased levels of TxB_2 above the basal level of 0.1 pmol/10^8 platelets over a 3 minute incubation period.

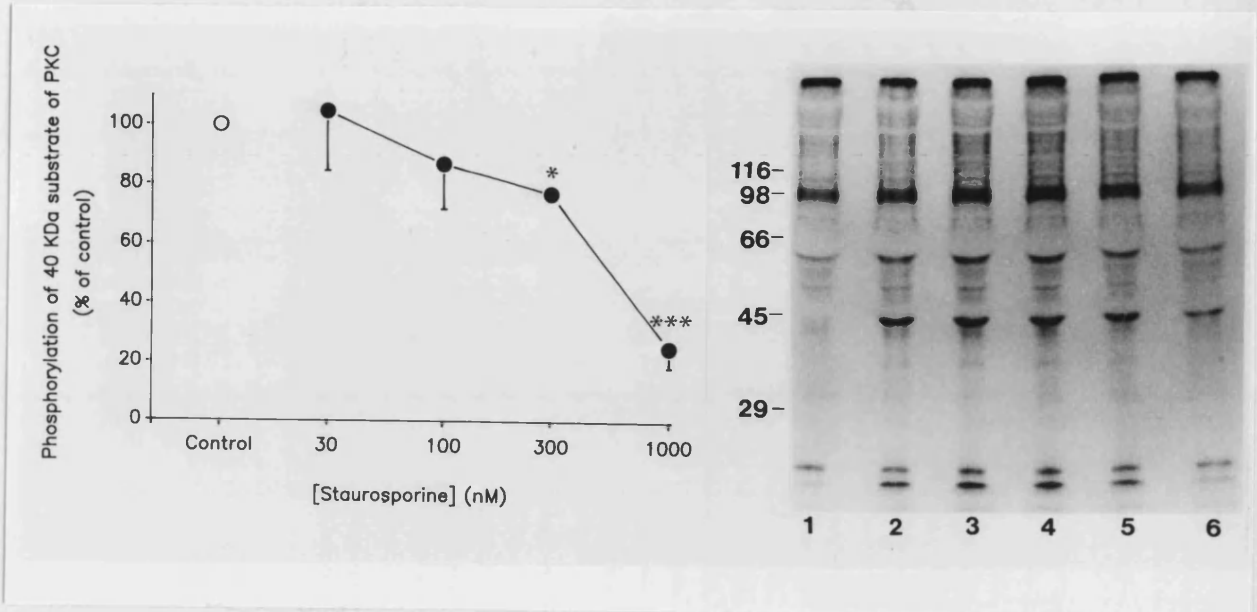


Fig 13 Inhibition of phosphorylation of the 40-47 kDa substrate of PKC by staurosporine in platelets. Right panel: autoradiograph representing track 1 = phosphorylation in resting platelets, track 2 = 300 nM PAF induced phosphorylation in the presence of staurosporine vehicle (0.01% DMSO) and tracks 3-6 = pretreatment with 30,100,300 & 1000 nM staurosporine respectively followed by 300 nM PAF stimulation. Platelet activation was terminated at 30s post-PAF. Left panel: Phosphorylation of the PKC substrate as a percentage of that phosphorylated by 300 nM PAF (in the presence of vehicle). Phosphorylation was quantified by scintillation counting of the excised $[^{32}P]P_i$ labelled PKC substrate.

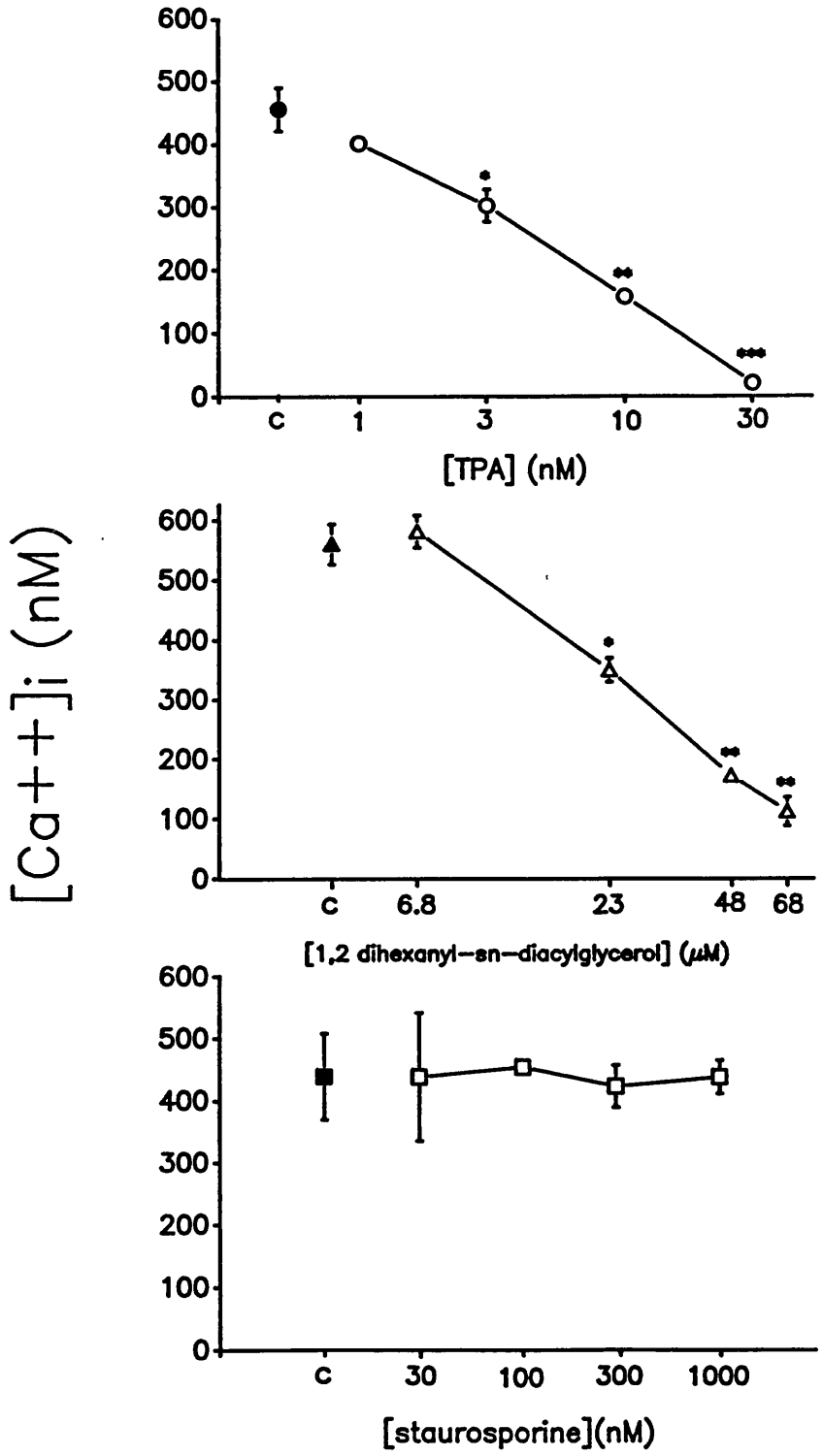


Fig 14 Effect of PKC modulation on $[Ca^{++}]_i$ elevation induced by PAF. Platelets were preincubated for 2 minutes with the PKC activators TPA (upper panel), sn-1,2-DHG (middle panel) or the PKC inhibitor staurosporine (lower panel) and then stimulated with 3 nM PAF. The $[Ca^{++}]_i$ value was obtained by subtracting the basal pre-PAF value from the peak post-PAF value. The values for each of the experiments are from at least 3 separate experiments each performed in triplicate.

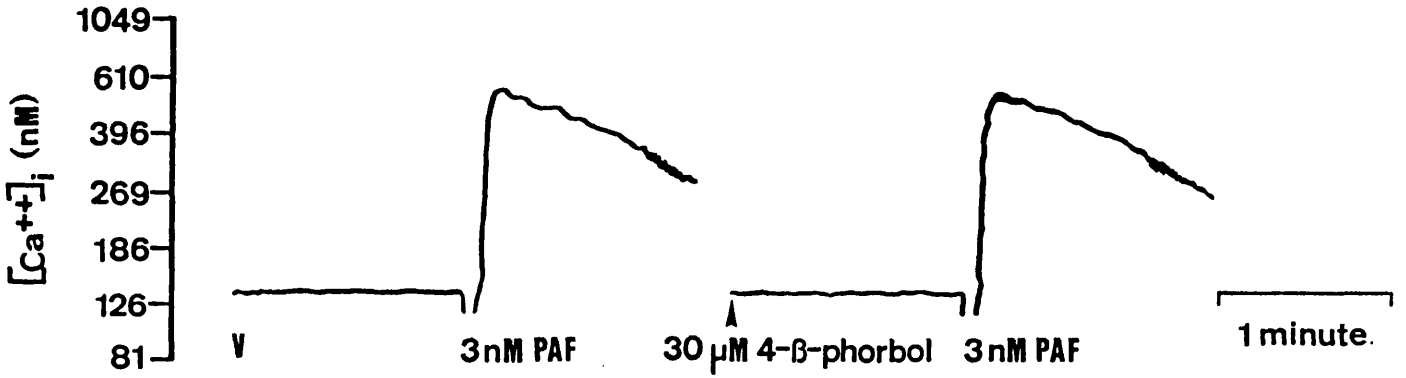


Fig 15 The effect of 4-ß-phorbol on PAF-induced $[Ca^{++}]_i$ elevation. Platelets were pretreated with 30 μM 4-ß-phorbol for two minutes before stimulation with 3 nM PAF. The traces are from a single experiment but are representative of two different experiments each performed in triplicate.

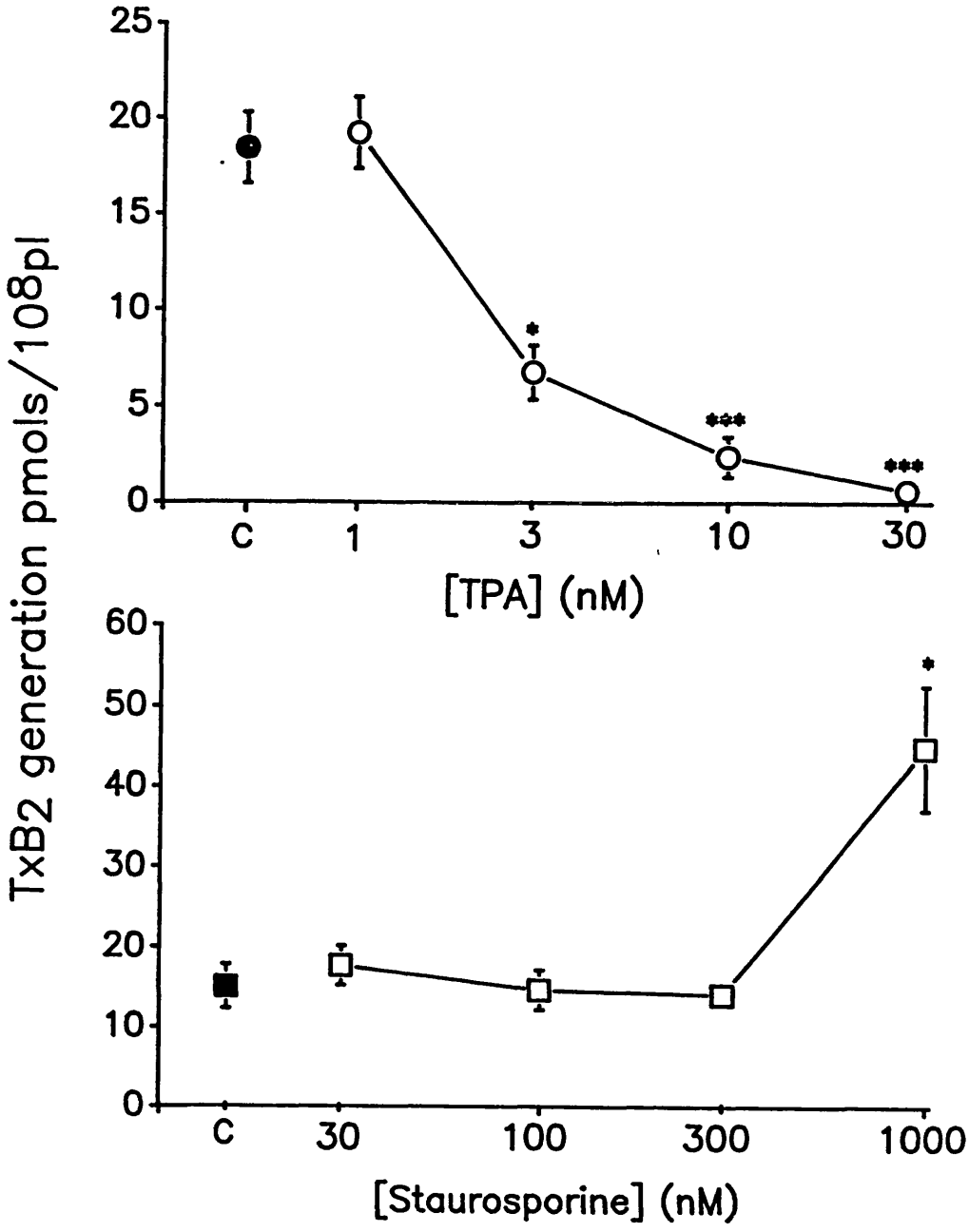


Fig 16 Effect of PKC modulation on TxB_2 generation induced by PAF. Platelets were pretreated for 2 minutes with the PKC activator, TPA (upper panel) or with staurosporine (lower panel) and then stimulated with 30 nM PAF. TxB_2 generation was determined at 3 minutes after PAF addition. The values are the mean ± S.E.M of three experiments each performed in triplicate.

The dependence of TxB_2 generation and 5-HT release on $[Ca^{++}]_i$ elevation was determined by replacing $[Ca^{++}]_i$ with the Ca^{++} chelator EGTA (Fig 17). Removal of $[Ca^{++}]_e$ caused an inhibition of $[Ca^{++}]_i$ elevation and both TxB_2 generation and 5-HT release induced by a range of concentrations of PAF (Fig 17), this would indirectly suggest that TxB_2 generation and 5-HT release are dependent on $[Ca^{++}]_i$ elevation.

The ability of staurosporine to inhibit phosphorylation of the major 40-47 kDa PKC substrate demonstrated it was causing inhibition of PKC. In order to demonstrate that both staurosporine and TPA are working at the level of PKC, the ability of staurosporine to inhibit the effect of TPA or sn-1,2-DHG on PAF-induced $[Ca^{++}]_i$ elevation is shown in Fig 18. The inhibition of $[Ca^{++}]_i$ elevation induced by 30 nM TPA (or 68 μM sn-1,2-DHG) was dose dependently abrogated by staurosporine in 3 nM PAF stimulated platelets (Fig 18) indicating that the inhibition of $[Ca^{++}]_i$ elevation by TPA and sn-1,2-DHG was probably mediated by PKC.

3.1.4 PKC modulation and ionomycin-induced platelet activation

In contrast to its effect on $[Ca^{++}]_i$ elevation induced by PAF, TPA had no effect on $[Ca^{++}]_i$ elevation in platelets stimulated with a submaximal concentration of ionomycin (Fig 19). Staurosporine also had no effect on ionomycin induced $[Ca^{++}]_i$ elevation (Fig 19). In addition, inhibition of the cyclo-oxygenase pathway with 2.8 μM of indomethacin also had no significant effect on $[Ca^{++}]_i$ elevation induced by 5 μM ionomycin (Fig 19), indicating that elevation of $[Ca^{++}]_i$ in ionomycin stimulated platelets was not dependent on TxA_2 generation. TxB_2 generation in ionomycin stimulated platelets was however modified by both PKC activation and inhibition. TPA (1-30 nM) caused a dose dependent potentiation of TxB_2 generation (Fig 20) whilst PKC inhibition with staurosporine caused a dose dependent inhibition of TxB_2 generation (Fig 20).

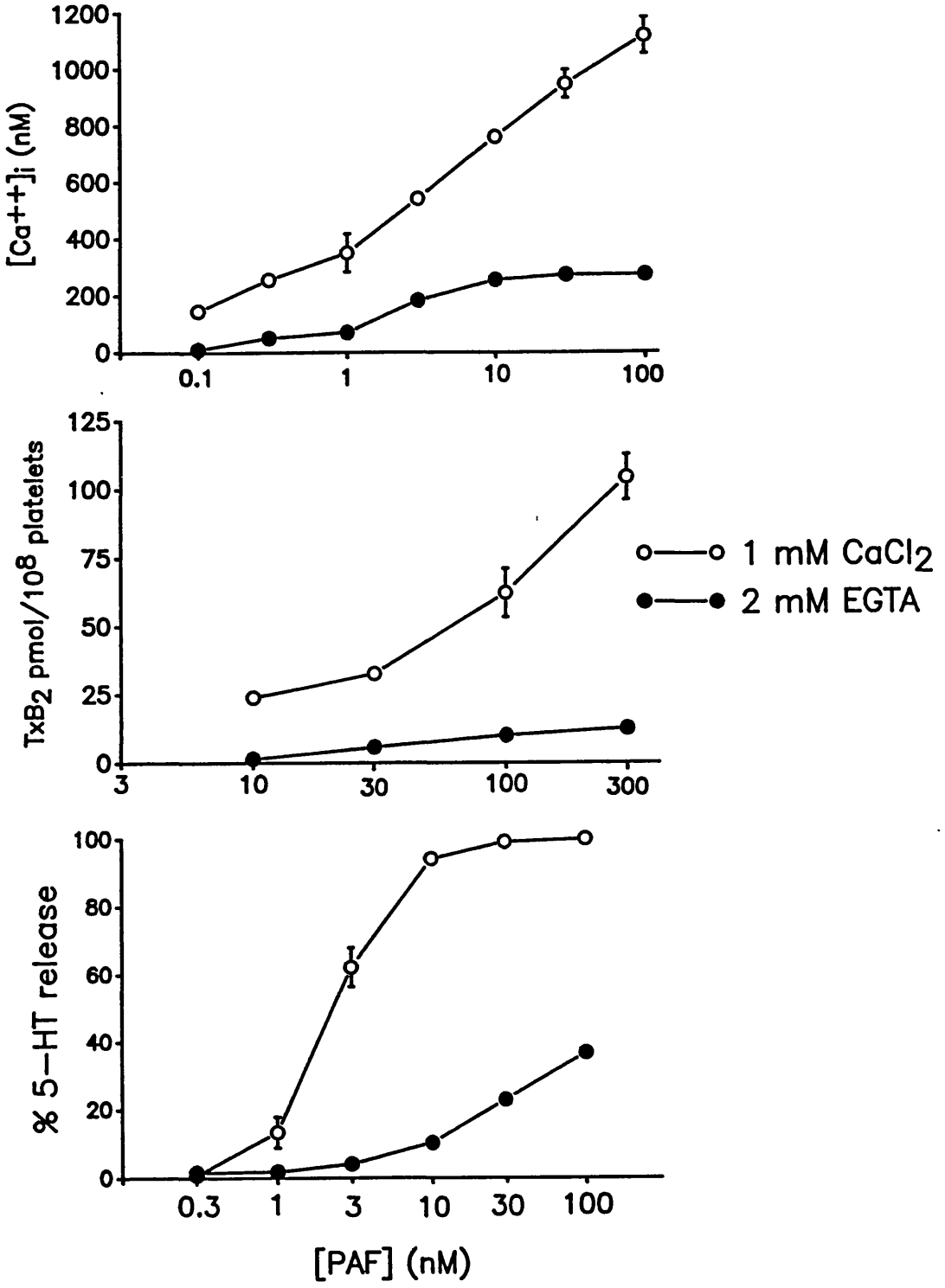


Fig 17 The effect of replacing 1 mM extracellular calcium with 2 mM EGTA on PAF-induced $[Ca^{++}]_i$ elevation (upper panel), TxB_2 generation (middle panel) and dense granule release (lower panel). $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-PAF value from the peak value for $[Ca^{++}]_i$ after platelet activation. TxB_2 generation and 5-HT release were determined 3 minutes after the addition of PAF. Open circles denote the presence of 1 mM $[Ca^{++}]_e$ and closed circles the presence of 2 mM $[EGTA]_e$. These values are the means ± S.E.M of three separate experiments each performed in triplicate.

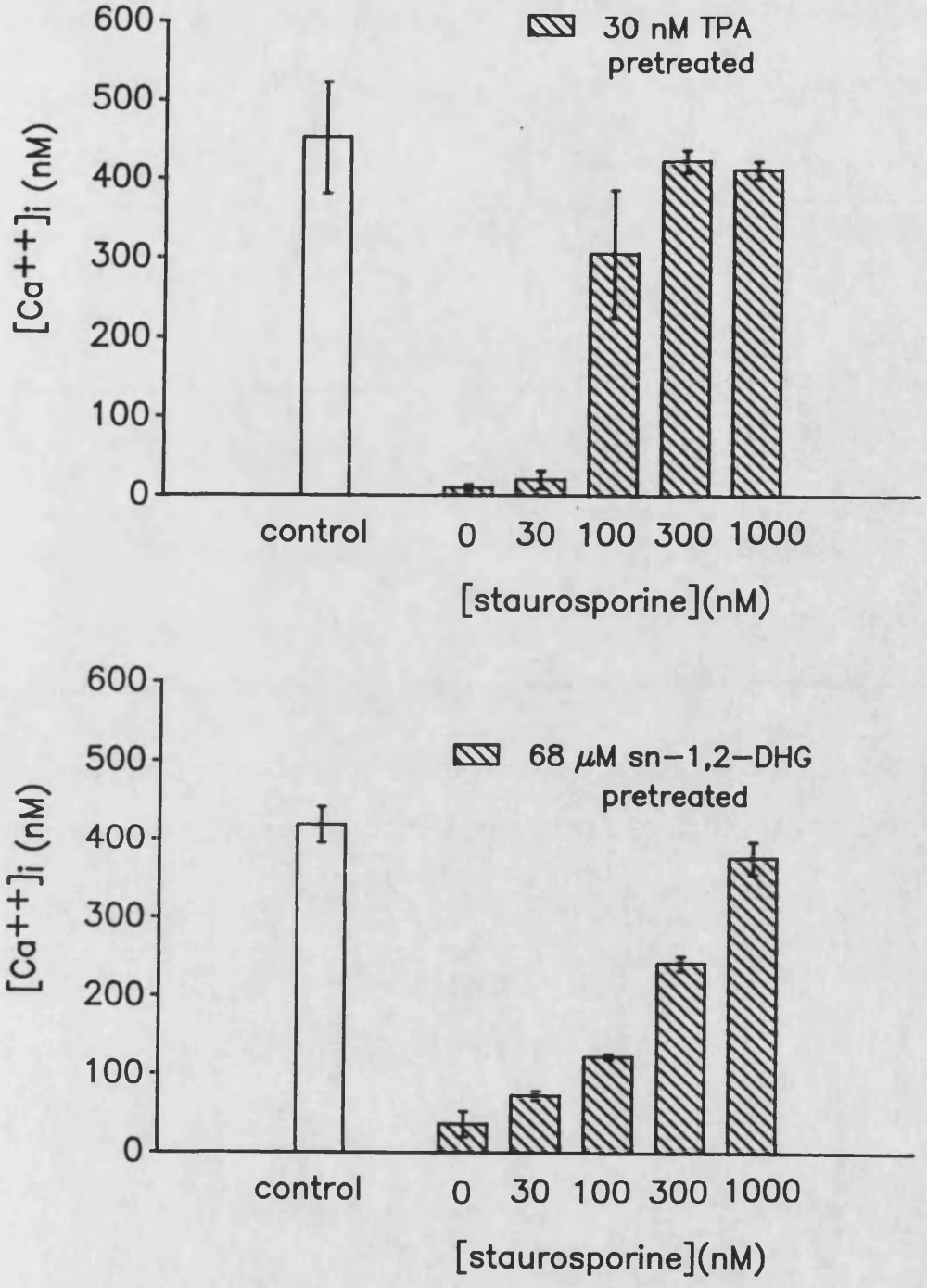


Fig 18 Reversal of TPA (upper panel) and sn-1,2-DHG (lower panel)-induced inhibition of $[Ca^{++}]_i$ elevation by staurosporine in 3 nM PAF-stimulated platelets. Staurosporine was added 2 minutes before TPA (30 nM) or sn-1,2-DHG (68 μM) which was added 2 minutes prior to stimulation with 3 nM PAF. $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-PAF value from the maximal post-PAF value. The values in these experiments are the mean ± S.E. from two separate experiments each performed in triplicate.

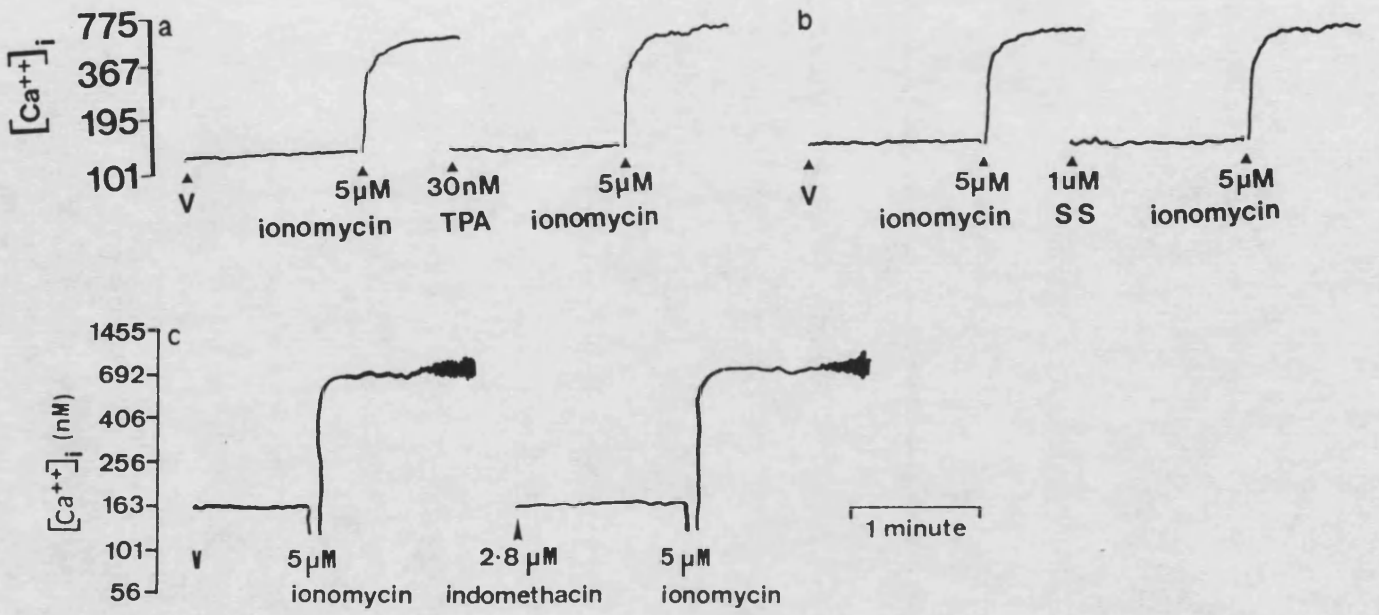


Fig 19 Effect of modulating PKC on ionomycin induced $[Ca^{++}]_i$ elevation. The traces represent the fluorescent output from fura-2 loaded platelets which have been pretreated for two minutes with (a) 30 nM TPA, (b) 1 μM staurosporine or (c) 2.8 μM indomethacin and then stimulated with 5 μM ionomycin. The traces are taken from a single experiment, but are representative of two other similar experiments.

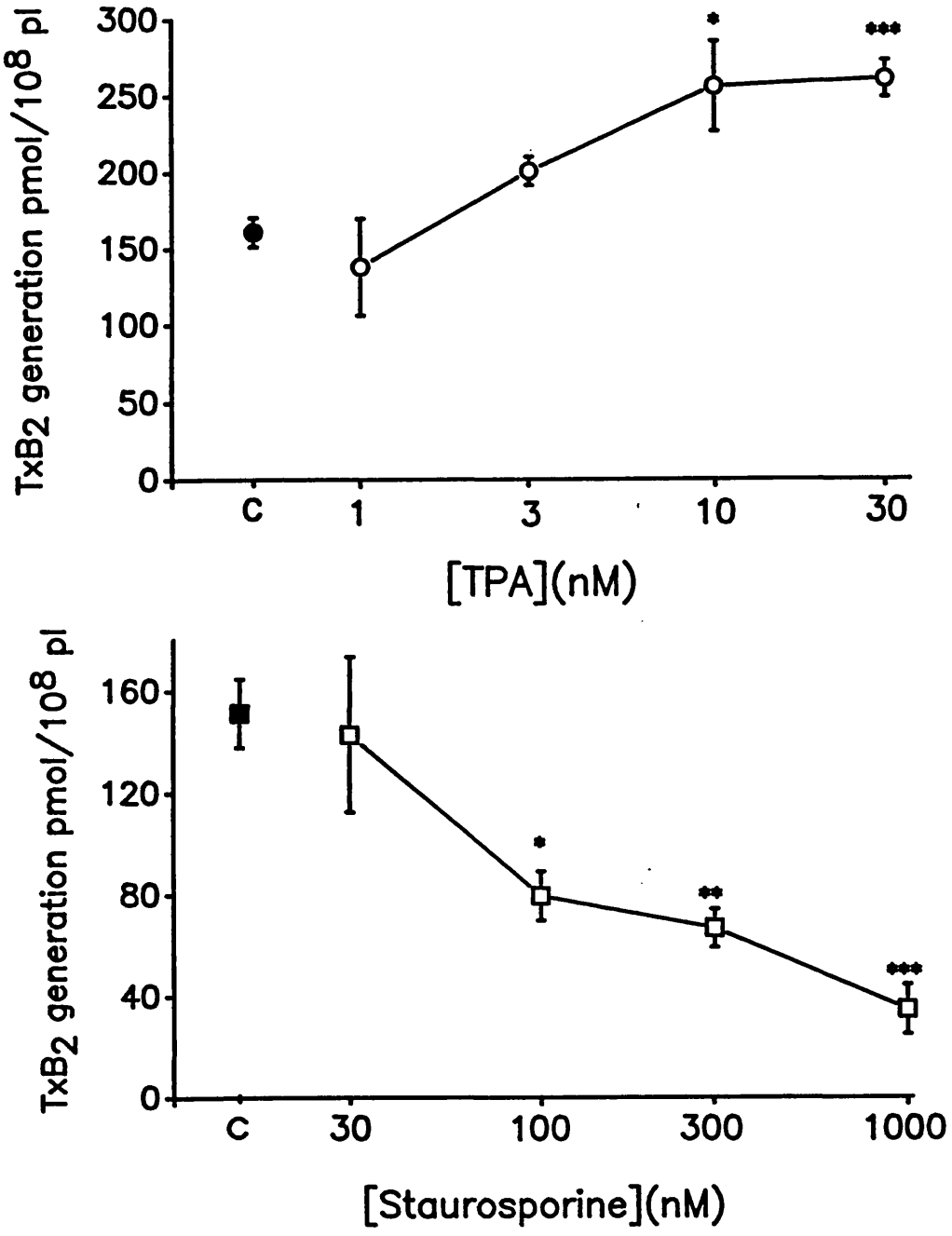


Fig 20 PKC modulation on 5 μM ionomycin-induced TxB_2 generation. Platelets were pretreated for 2 minutes with either TPA (upper panel) or staurosporine (lower panel) before stimulation with 5 μM ionomycin. TxB_2 generation was determined 3 minutes post-ionomycin stimulation. The values are the mean ± S.E.M. of three separate experiments each performed in triplicate.

Although 5 μM ionomycin was a submaximal concentration for cell functional responses it was important to establish that this concentration of ionomycin was not causing non-specific cell permeabilization or cell lysis. Therefore the supernatant of ionomycin stimulated cells was examined for lactate dehydrogenase activity which is a marker for cell damage. The supernatant was also examined for low molecular weight molecule leakage by testing for leakage of fura-2 from the cells. At 5 μM ionomycin, there was no lactate dehydrogenase activity in the supernatant nor did any fura-2 leak from the cells into the extracellular medium until a concentration of above 50 μM ionomycin was added to the platelets.

3.1.5 PKC modulation and arachidonic acid-induced platelet activation

In agreement with the findings for PAF stimulated platelets, pretreatment of platelets with TPA caused a dose dependent inhibition of $[Ca^{++}]_i$ elevation whilst staurosporine had no effect on $[Ca^{++}]_i$ elevation in platelets stimulated with AA (Fig 21). Neither PKC activation nor inhibition had any effect on TxB_2 generation in platelets stimulated with AA (Fig 22). A concentration of 30 μM AA was found to be submaximal for both $[Ca^{++}]_i$ elevation and TxB_2 generation. It was important to establish that 30 μM AA was not causing non-specific cell permeabilization or lysis, therefore the supernatant of AA stimulated platelets was examined for lactate dehydrogenase activity and for fura-2 leakage from the cells upon AA stimulation. No lactate dehydrogenase activity was detected in the supernatant nor did any fura-2 leak into the extracellular medium of platelets stimulated with 30 μM AA.

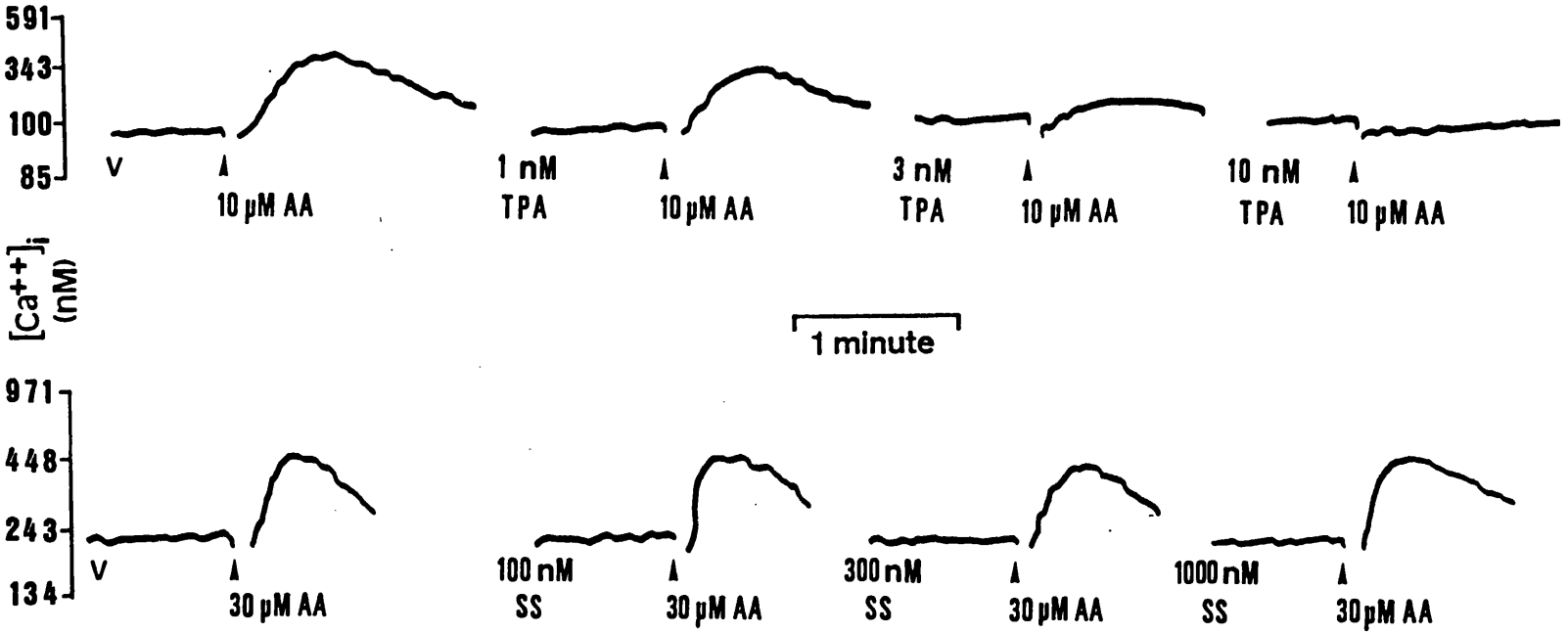


Fig 21 PKC modulation on arachidonic acid-induced $[Ca^{++}]_i$ elevation. The traces represent the fluorescent output from fura-2 loaded platelets which have been pretreated for two minutes with a dose range of TPA then stimulated with 10 μM arachidonic acid (a) or a dose range of staurosporine and then stimulated with 30 μM arachidonic acid (b). The traces are taken from a single experiment which was representative of two similar experiments each performed in triplicate.

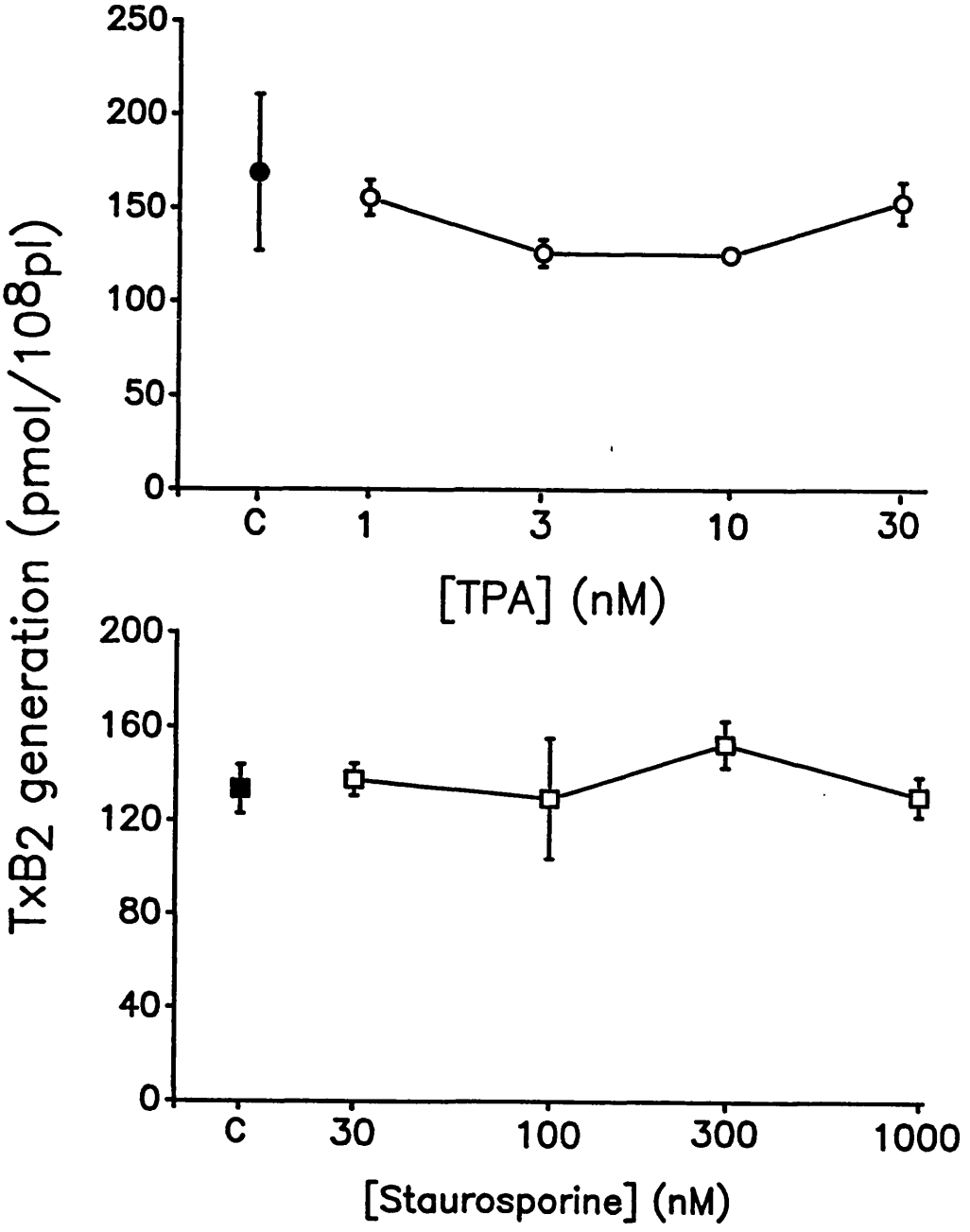


Fig 22 PKC modulation on arachidonic acid-induced TxB_2 generation. Platelets were pretreated for 2 minutes with either TPA (upper panel) or staurosporine (lower panel) and then stimulated with 30 μM arachidonic acid. TxB_2 generation was measured at 3 minutes post-stimulation. The results are taken from a single experiment which is representative of two other similar experiments.

3.1.6 PKC modulation on PAF and ionomycin-induced 5-HT release

Washed platelets released 5-HT from their dense granules in response to stimulation with either PAF or ionomycin (Fig 23). Activation of PKC with TPA alone also caused a small amount of dense granule release, with up to 6 percent of 5-HT released by addition of 30 nM TPA. In platelets stimulated with submaximal PAF (3 nM) activation of PKC with 1 - 30 nM TPA had no significant effect on 5-HT release (Fig 23a). Staurosporine however inhibited 5-HT release with an IC_{50} of 800 nM, causing up to 70 percent inhibition with 1 μM staurosporine (Fig 23b).

In ionomycin stimulated platelets TPA caused a significant potentiation of 5-HT release with 100% release at 30 nM TPA (Fig 23a). This potentiation however was not due to the increased generation of TxA_2 caused by TPA in ionomycin stimulated platelets (see Fig 20) as inhibition of TxA_2 generation with 2.8 μM indomethacin did not inhibit the potentiation of 5-HT release caused by TPA in ionomycin stimulated platelets (Fig 24). In contrast, staurosporine caused a significant inhibition of 5-HT release with an IC_{50} of 150 nM, and produced almost 100% inhibition at only 300 nM staurosporine (Fig. 23b). Staurosporine was therefore a weaker inhibitor of 5-HT release in platelets stimulated via a receptor operated mechanism (PAF) than in platelets directly activated via Ca^{++} elevation (ionomycin).

In order to assess whether loading platelets with the fluorescent dye fura-2 was causing a significant chelation of cytosolic Ca^{++}, 5-HT release experiments were investigated in platelets either loaded with fura-2 or in platelets not loaded with fura-2 (Fig 23). Fura-2 loading had no effect on 5-HT release in either PAF or ionomycin stimulated platelets, indicating that fura-2 was not chelating cytosolic Ca^{++} to an extent where Ca^{++}-dependent functional responses were suppressed.

Table 2 gives a summary of the effects of staurosporine and TPA on PAF, ionomycin and AA-induced $[Ca^{++}]_i$ elevation, dense granule release and TxB_2 generation.

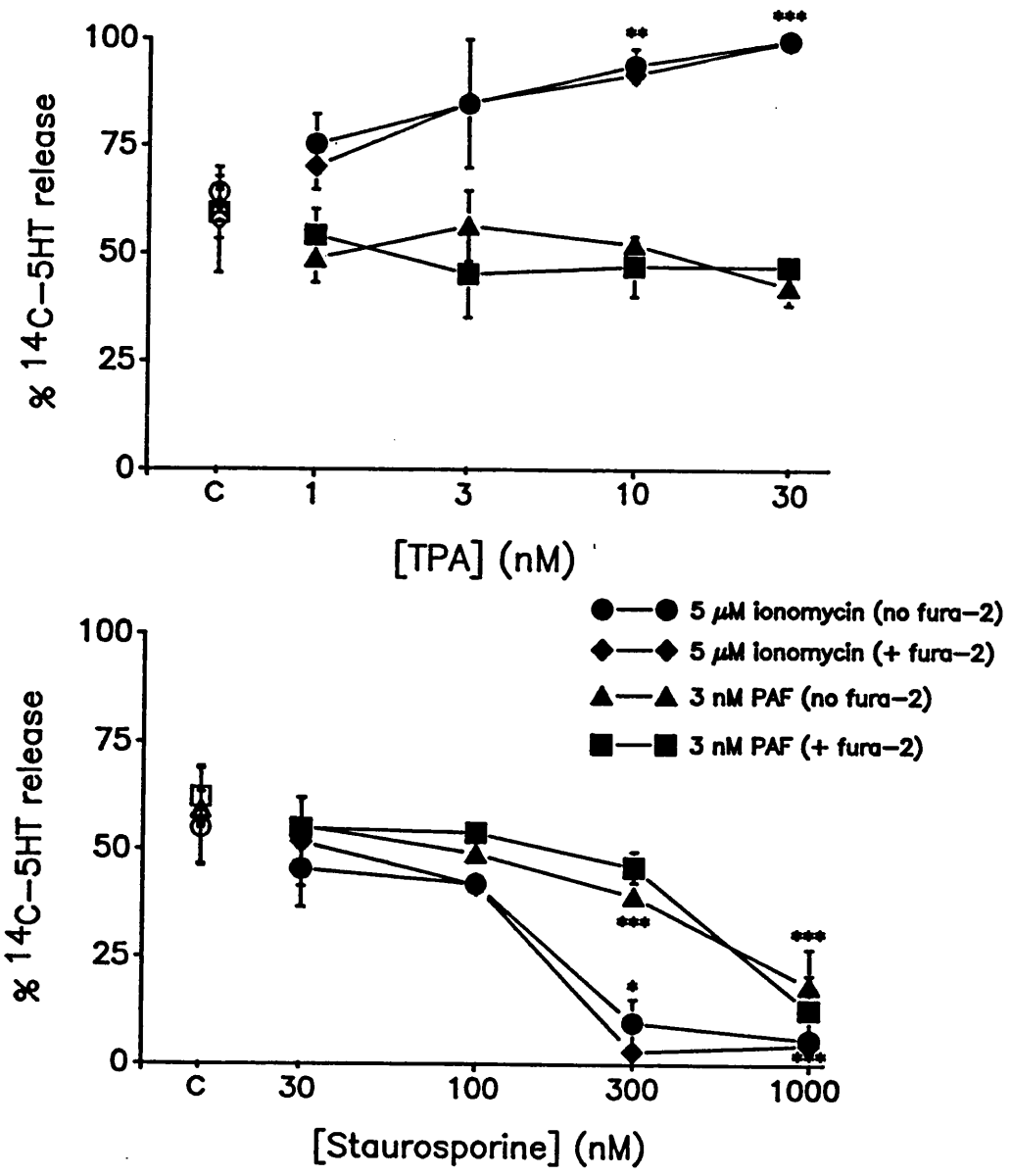


Fig 23 The effect of (a) TPA or (b) staurosporine on PAF or ionomycin-induced 5HT release in platelets either loaded or not loaded with fura-2. Platelets either loaded with fura-2 or not loaded were pre-incubated for 2 minutes with 1-30 nM TPA (or TPA vehicle) or 30 -1000 nM staurosporine (or staurosporine vehicle) before the addition of either 3 nM PAF or 5 μM ionomycin. In all cases 5-HT release was terminated 3 minutes after platelet activation. The significant differences shown are due to a statistically significant difference between 5-HT release from platelets treated with the vehicle control compared to treatment with a particular concentration of either TPA or staurosporine in platelets not loaded with fura-2. The points represent the means ± S.E.M. of 3 experiments each performed in triplicate.

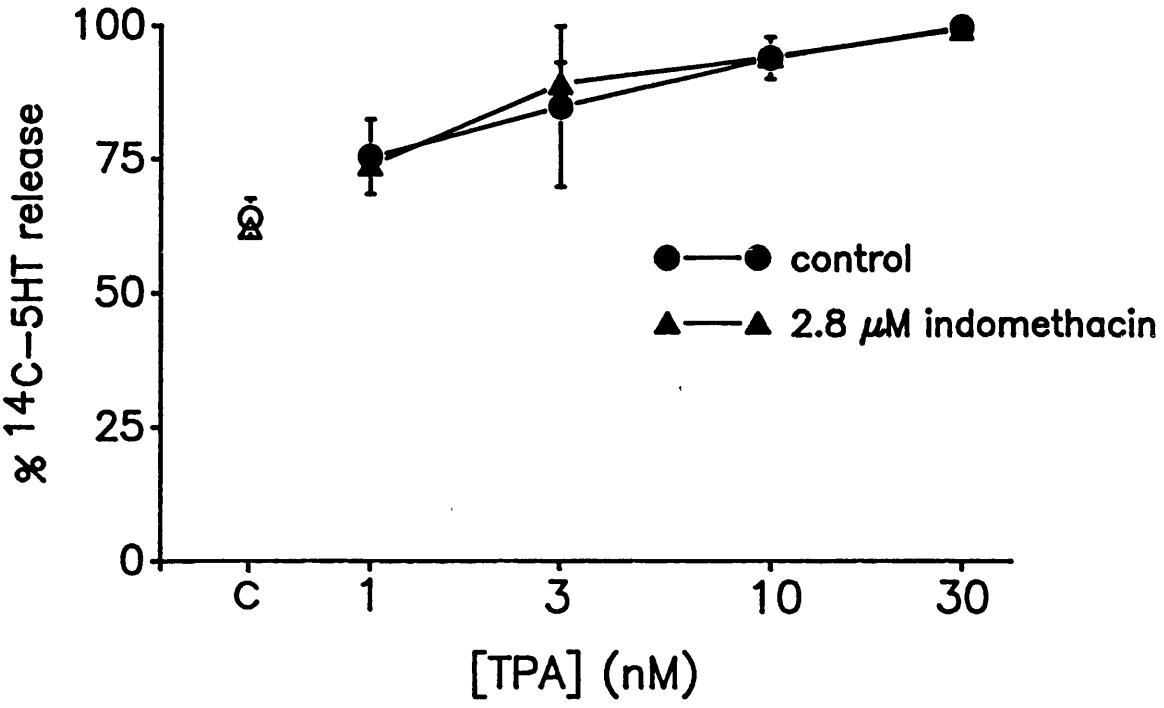


Fig 24 The effect of indomethacin on ionomycin-induced 5-HT release in platelets pre-treated with TPA. Platelets were pretreated with 2.8 μM indomethacin 2 minutes before the addition of TPA vehicle or increasing concentrations of TPA (1-30 nM) which were added 2 minutes prior to stimulation with 5 μM ionomycin. In all cases 5-HT release was terminated 3 minutes post-ionomycin. The points represent the means ± S.E.M. of 3 experiments each performed in triplicate.

	PAF		IONOMYCIN		ARACHIDONIC ACID	
	SS	TPA	SS	TPA	SS	TPA
Calcium elevation	↑($t_{1/2}$)	↓(Ht)	ND	ND	↑($t_{1/2}$)	↓(Ht)
TxB_2 generation	↑	↓	↓	↑	ND	ND
5-HT release	↓	ND	↓	↑	-	-

- = not determined
ND = not different

↓ decrease
↑ increase

Table 2 Summary of the effects of staurosporine or TPA on PAF, ionomycin and arachidonic acid induced $[Ca^{++}]_i$ elevation, TxB_2 generation and dense granule release.

3.1.7 Inhibition of PKC on PAF-induced platelet desensitization

It is known that upon stimulation with PAF, platelets are specifically desensitized and lose their ability to become activated by a further exposure to PAF (Keraly and Benveniste, 1982). Platelets are however still capable of being activated by other agonists binding at different receptor sites. It has been demonstrated in this study that activation of PKC inhibits PAF-induced $[Ca^{++}]_i$ elevation (Fig 14) but that this inhibition can be reversed by pre-incubation with the PKC inhibitor staurosporine (Fig 18). It is possible therefore that PKC activation is responsible for the desensitization of platelets to PAF once they have been stimulated by PAF and if so inhibition of PKC with staurosporine may prevent this desensitization.

Stimulation of platelets with either 0.3, 3 or 30 nM PAF caused a transient elevation in $[Ca^{++}]_i$ which was allowed to decline to near basal levels before the addition of a second dose of PAF at the same concentration as the first. The second dose of PAF caused a much smaller increase in $[Ca^{++}]_i$ than the first dose, for example, addition of 3 nM PAF caused an elevation of 450 nM $[Ca^{++}]_i$ whereas the second dose caused an elevation of only 20 nM. Peak $[Ca^{++}]_i$ elevation induced by the second dose of PAF was inversely proportional to the concentration of the first dose of PAF added, such that the peak $[Ca^{++}]_i$ elevation induced by the second dose of 30 nM PAF was smaller than the peak $[Ca^{++}]_i$ elevation induced by the second dose of 0.3 nM PAF. This may indicate that at the lower concentration of PAF (0.3 nM) not all PAF receptors were desensitized therefore allowing $[Ca^{++}]_i$ elevation when the platelets were stimulated with a second dose of PAF. Inhibition of PKC with 300 nM staurosporine however did not prevent desensitization of PAF-induced $[Ca^{++}]_i$ elevation to further stimulation by PAF at any of the concentrations of PAF employed (Fig 25).

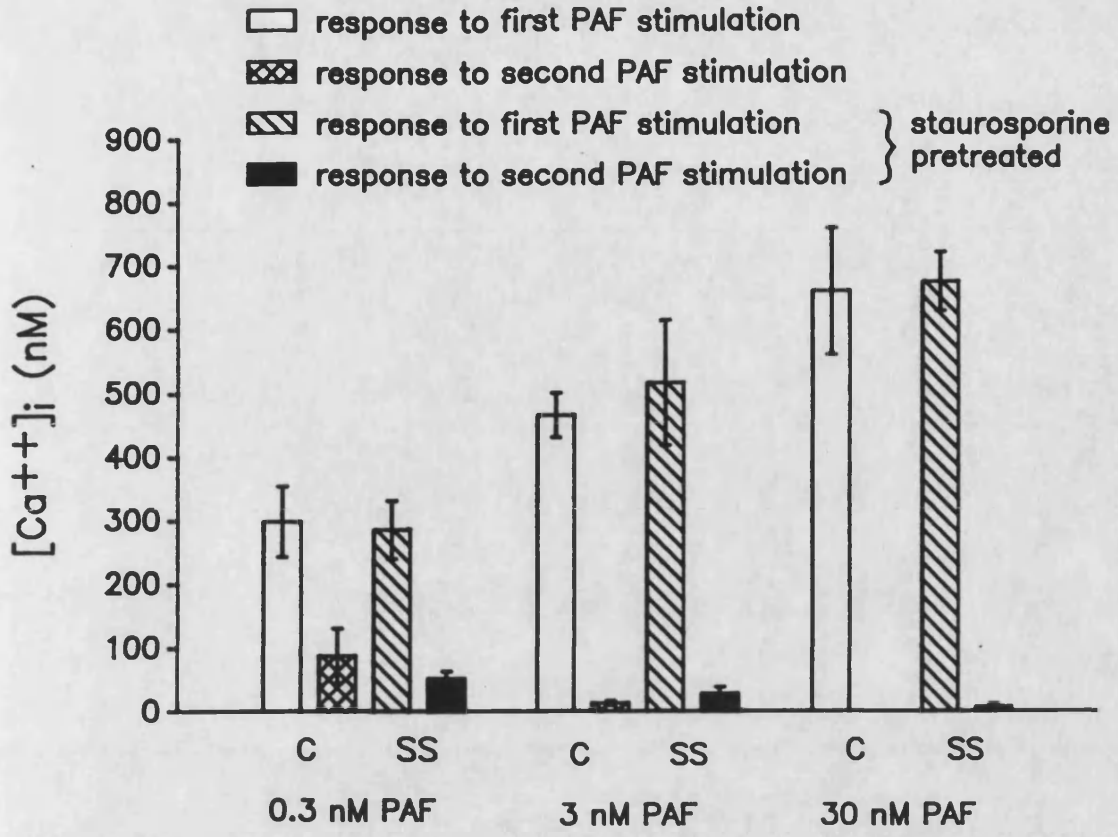


Fig 25 Effect of staurosporine on PAF-induced desensitisation. Platelets were either pretreated with 1 μM staurosporine or staurosporine vehicle two minutes before addition of the first dose of PAF (0.3, 3 or 30 nM) and a second dose of the same concentration of PAF was added when $[Ca^{++}]_i$ levels reached near basal levels. The values are the mean ± S.E. of two experiments each performed in triplicate.

3.2 Characterisation of signal molecule elevation in PAF stimulated platelets and investigation of the role of PKC on signal molecule production

3.2.1 PAF-induced elevation of the concentration of $[Ca^{++}]_i$, Ins(1,4,5)P_3 and DAG

Addition of 300 nM PAF induced a rapid elevation of platelet $[Ca^{++}]_i$ from a basal concentration of 152 ± 11 nM (n=30) to a peak elevation of 1023 ± 72 nM at 7.3 ± 0.6s (n=13) post-PAF. This elevation was transient and quickly declined (Fig 26). Similarly, in the same platelet preparations 300 nM PAF induced a rapid elevation in the concentration of Ins(1,4,5)P_3 with a peak increase at 2s of 38.9 ± 2.2 pmol/10^9 platelets above a basal level of 9.77 ± 1.72 pmol/10^9 platelets (Fig 26). This elevation rapidly declined to basal levels by 15s after PAF, followed by a gradual increase at 30 and 60s post PAF.

Superimposition of the Ins(1,4,5)P_3 and $[Ca^{++}]_i$ time curves demonstrates that the Ins(1,4,5)P_3 peak clearly precedes the peak $[Ca^{++}]_i$ by approximately 5 seconds.

Platelets had a basal concentration of 117 ± 3 pmol of DAG in 10^9 platelets. Following the addition of 300 nM PAF, there was a rapid and transient increase in the platelet DAG concentration which peaked at 10s with a value of 280 ± 52 pmol/10^9 platelets (n=9) above basal. After 10 s the concentration of DAG rapidly declined and by 15s reached a level above basal at which it remained for the duration of the time course (60s) (Fig 26).

Addition of PAF vehicle did not produce changes in the basal concentration of $[Ca^{++}]_i$, Ins(1,4,5)P_3, or DAG.

Table 3 demonstrates that pretreatment of the platelets with the PAF receptor antagonist WEB 2086 (300 nM) inhibited to the same degree PAF-induced elevation of Ins(1,4,5)P_3 and DAG.

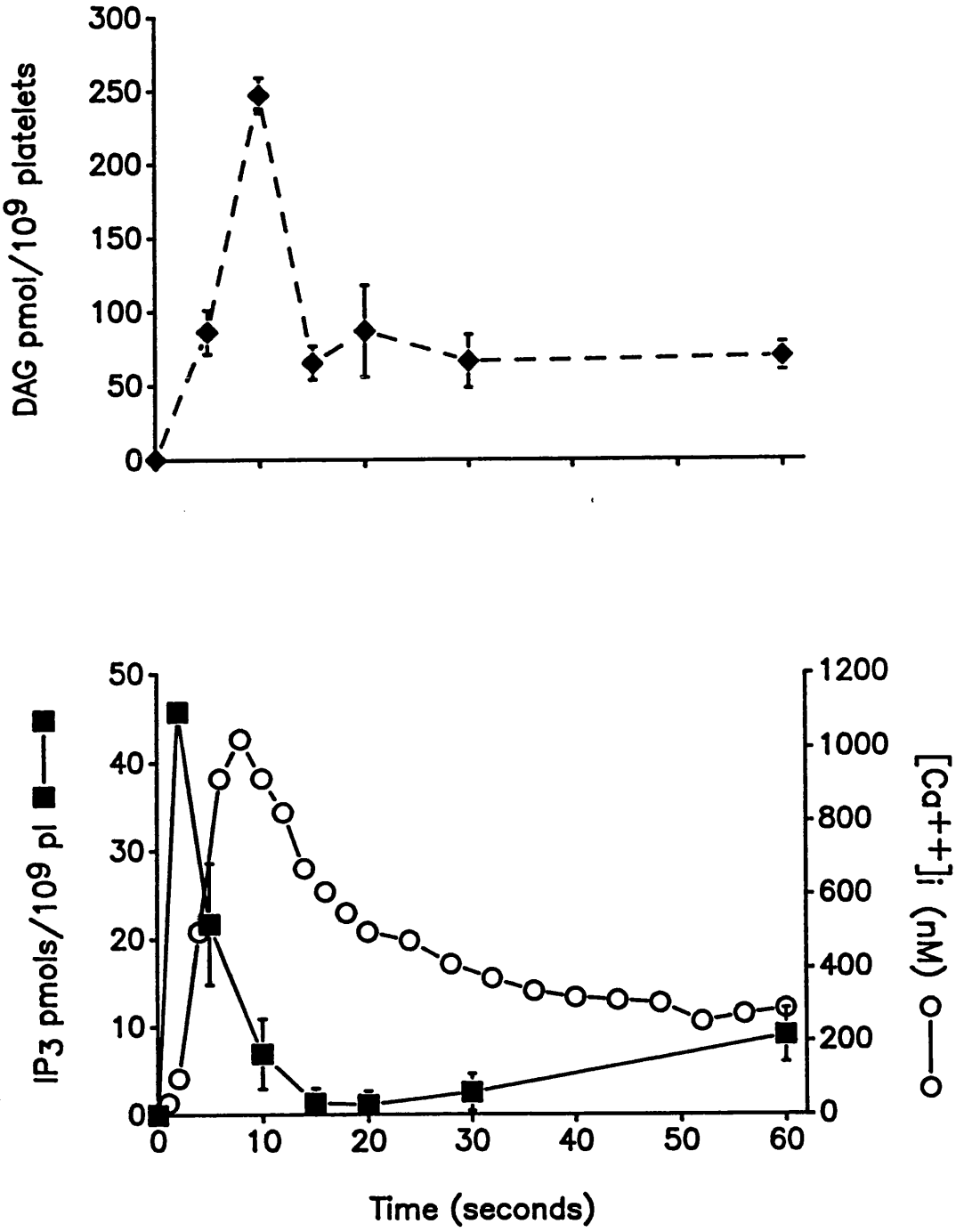


Fig 26 Time courses for the production of DAG, Ins(1,4,5)P_3 and $[Ca^{++}]_i$ in 300 nM PAF-stimulated platelets. DAG production induced by 300 nM PAF is shown in the upper panel, and cytosolic calcium ($[Ca^{++}]_i$) elevation together with Ins(1,4,5)P_3 elevation are shown in the lower panel. The values in all figures have been obtained by subtracting the basal pre-PAF value from the values obtained at the stated times after 300 nM PAF administration. The values are the mean ± S.E.M. of at least three experiments. The S.E.M. bars are absent when symbols are larger than the S.E.M.

Treatment	Generation (% control) Ins(1,4,5)P_3 *	DAG**
Vehicle + 300 nM PAF	100	100
30 nM WEB 2086 + 300 nM PAF	24.0 ± 14	22.5 ± 16
300 nM WEB 2086 + 300 nM PAF	4.7 ± 3.2	0

* measured 5s after PAF addition

** measured 10s after PAF addition

Table 3 Effect of a 2 minute pre-incubation with the PAF receptor antagonist WEB 2086 on PAF-induced Ins(1,4,5)P_3 and DAG generation.

In order to establish that PAF was not itself being converted into [^{32}P]-phosphatidic acid two control experiments were performed. In the first experiment different concentrations of PAF were added to the DAG standards and the resultant [^{32}P]PA produced was compared to normal standards not 'spiked' with PAF. Addition of PAF to standards had no effect on the final [^{32}P]PA count obtained indicating that PAF could not be directly converted into phosphatidic acid (PA). Secondly, platelets were stimulated with [^{3}H]PAF, the DAG produced was then converted into PA using DAG kinase, but instead of using [^{32}P]-labelled ATP, non-radiolabelled ATP was used. Because of the position of the radiolabel ([1-O-[^{3}H]Octadecyl-2-acetyl-sn-glycerol-3-phosphocholine) then any PAF which was eventually converted to PA would produce [^{3}H]PA. Scintillation counting showed no [^{3}H] contained in the PA derived from [^{3}H]PAF stimulated platelets. This indicated that the PAF used to stimulate the platelets was not being converted into DAG and ultimately not producing PA.

3.2.2 Dose-response relationship of PAF-induced platelet activation

The relationship of the concentrations of the three putative signal molecules, namely $[Ca^{++}]_i$, Ins(1,4,5)P_3 and DAG to the release of 5-HT was determined (Fig 27).

PAF is a powerful dense granule releasing agent and the dose-response relationship is shown in the lower panel of Fig 27. The higher doses of PAF, 30 and 300 nM which are maximal and supra-maximal for 5-HT release, produced highly significant ($P<0.005$) elevations in $[Ca^{++}]_i$, in addition to a rise in the concentration of Ins(1,4,5)P_3 (measured at 5s) and DAG (measured at 10s) (Fig 27).

However, a lower concentration of PAF, such as 3 nM which induced approximately 60 percent 5-HT release and a 400 nM increase above the basal $[Ca^{++}]_i$, induced no detectable increase in either Ins(1,4,5)P_3 or DAG (Fig 27). Indeed, over a time course of 60 s no increase in Ins(1,4,5)P_3 levels above basal were detected in platelets stimulated with 3 nM PAF (results not shown).

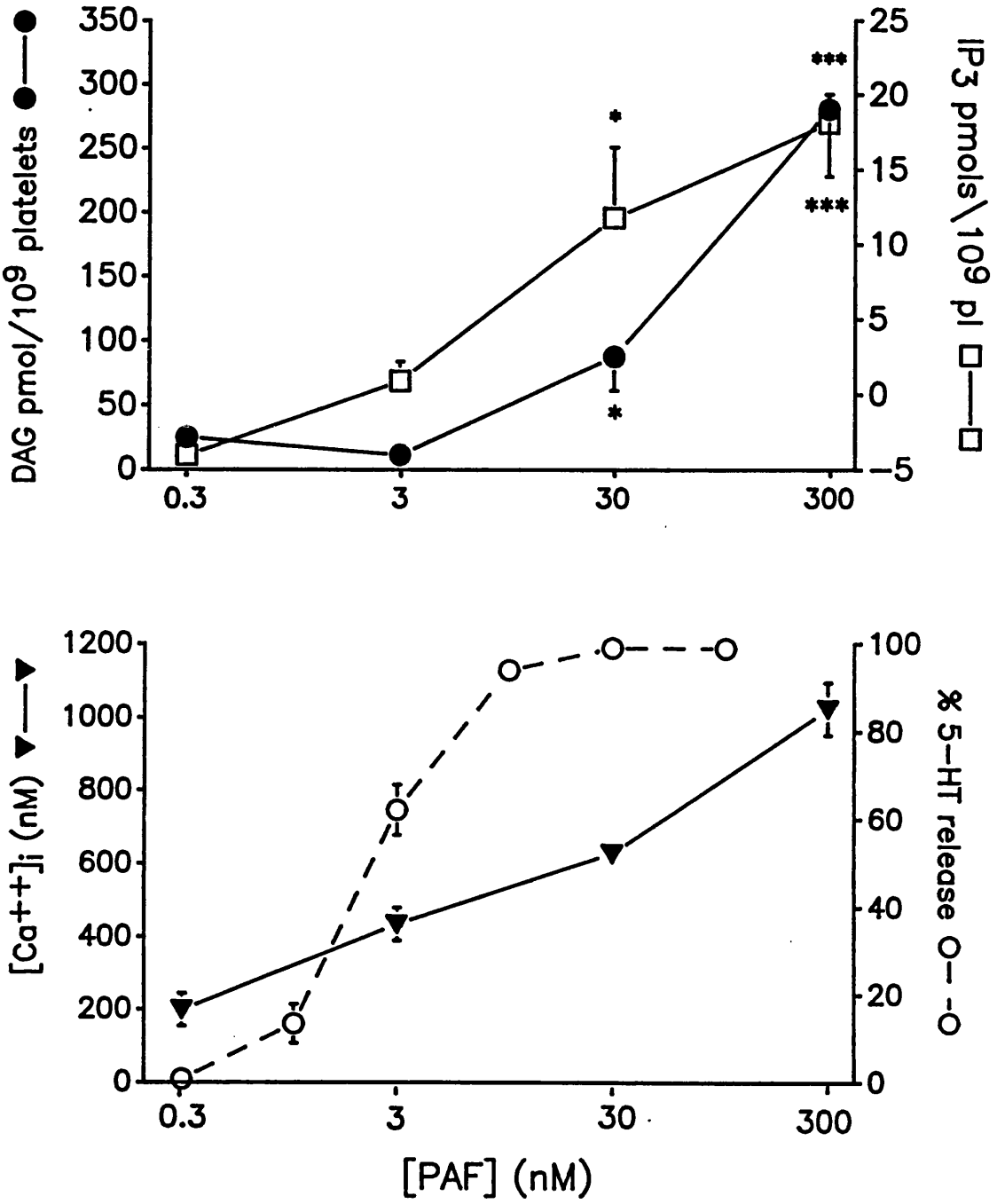


Fig 27 Effect of increasing concentrations of PAF on production of DAG, Ins(1,4,5)P_3 (upper panel), $[Ca^{++}]_i$ elevation and 5-HT release (lower panel). DAG incubations were terminated at 10s after PAF and those for Ins(1,4,5)P_3 at 5s. 5-HT release was terminated at 3 minutes after activation and $[Ca^{++}]_i$ elevation is peak elevation after stimulation. The values have been obtained by subtracting the basal pre-PAF value from those obtained after the addition of PAF. The values are the mean ± S.E.M. of at least 3 experiments.

Replacing $[Ca^{++}]_i$ with the Ca^{++} chelator EGTA inhibited $[Ca^{++}]_i$ elevation at all concentrations of PAF tested (Fig 17). At the higher concentrations of PAF, 30 and 300 nM, and to a lesser extent 3 nM, $[Ca^{++}]_i$ elevation was due to two components, the major component being dependent on extracellular Ca^{++}, whilst the minor component was independent of $[Ca^{++}]_e$. At the lower concentration of 0.3 nM PAF however, $[Ca^{++}]_i$ elevation was completely dependent on the presence of $[Ca^{++}]_e$ (Fig 17).

Replacing $[Ca^{++}]_e$ with the Ca^{++} chelator EGTA also caused a small inhibition of $Ins(1,4,5)P_3$ production when examined over a 60 s time course (Fig 28). Removing $[Ca^{++}]_e$ reduced basal levels of $Ins(1,4,5)P_3$ and at 5 s after platelet activation with 300 nM PAF $Ins(1,4,5)P_3$ levels were 75 % of those for platelets in the presence of 1 mM $[Ca^{++}]_e$. After reaching maximal, $Ins(1,4,5)P_3$ levels in platelets either in the presence and absence of $[Ca^{++}]_e$ declined towards basal and by 10 seconds after activation there was no difference between the level of $Ins(1,4,5)P_3$ in platelets either in the presence or absence of $[Ca^{++}]_e$ (Fig 28). In order to ensure that the presence of EGTA *per se* was having no influence on the $Ins(1,4,5)P_3$ binding assay, a standard curve was performed in the presence of the same concentration of EGTA as would be contained in experimental samples. There was no difference between a standard curve performed in the presence of EGTA compared to one performed in the absence of EGTA. This indicated that the presence of EGTA was not affecting the binding assay.

In relation to the findings for DAG (Fig 27), activation of PKC measured as phosphorylation of its 40 kDa substrate was increased dose dependently with increasing concentrations of PAF; 3 nM PAF induced some activation of PKC after 30s, but again in agreement with the findings for DAG, no phosphorylation with 0.3 nM PAF was detected (Fig 29).

These results suggest that there is a good correlation with the products of $PtdIns(4,5)P_2$ hydrolysis and Ca^{++} mobilization with granule release at higher concentrations, but not at lower concentrations of PAF.

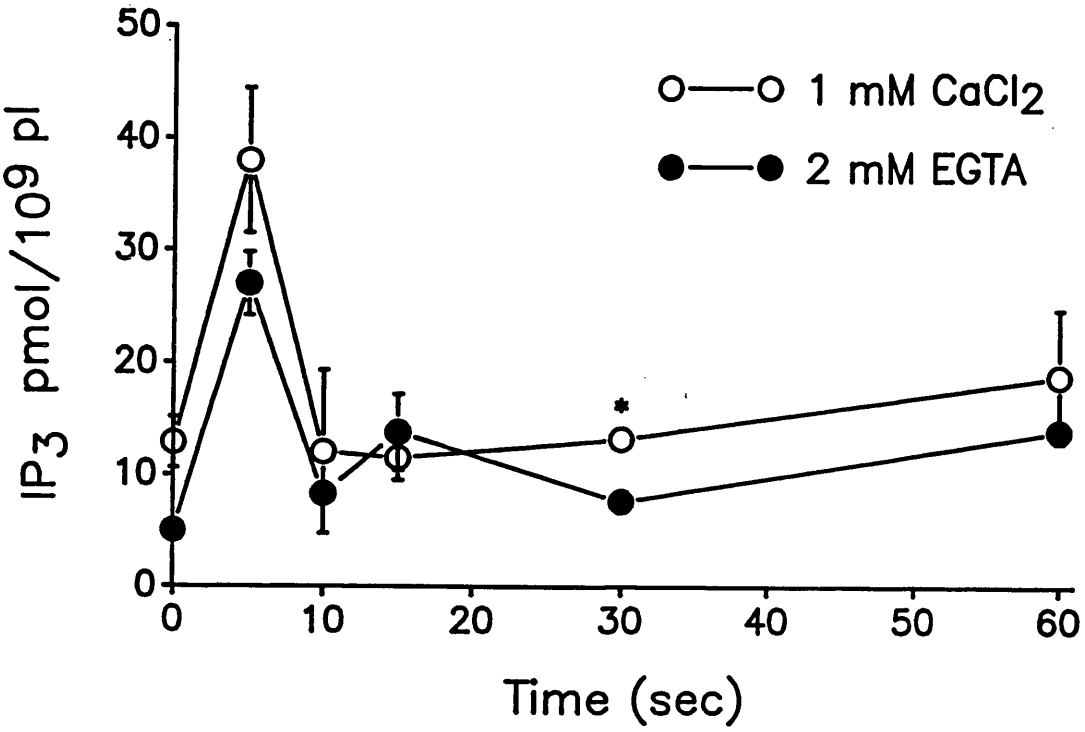


Fig 28 The effect of replacing $[Ca^{++}]_e$ with 2 mM of the calcium chelator EGTA on Ins(1,4,5)P_3 production induced by 300 nM PAF. Ins(1,4,5)P_3 production was stopped at predetermined times over a one minute time course. The values are the mean ± S.E.M. of 4 separate experiments each performed in duplicate.

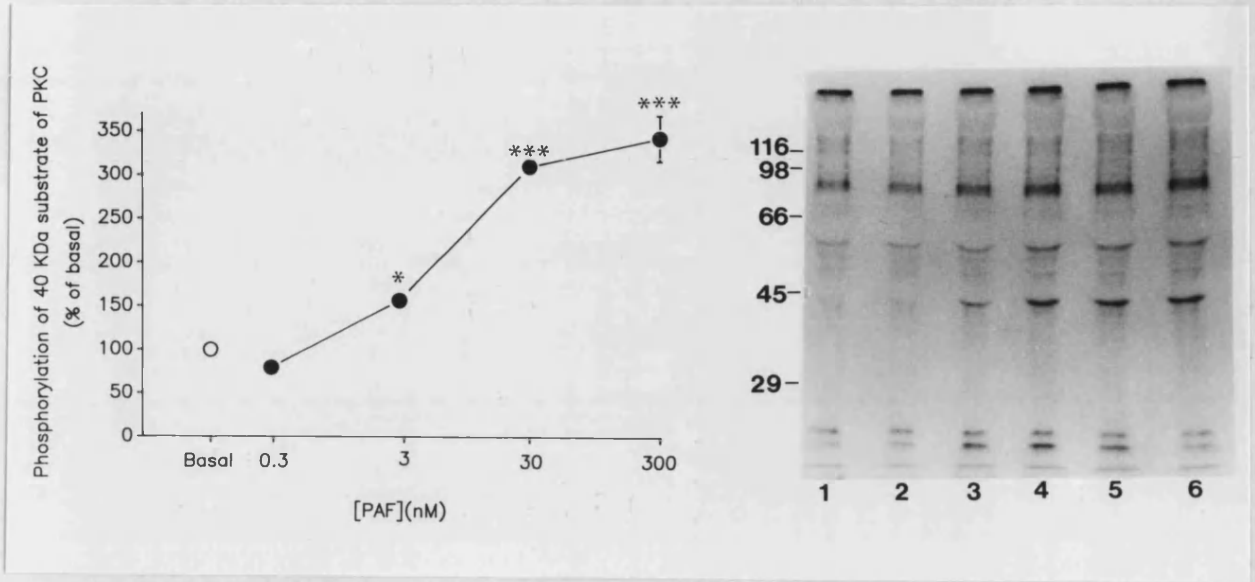


Fig 29 The effect of increasing concentrations of PAF on phosphorylation of the 40 KDa substrate of PKC. (a) Autoradiograph showing phosphorylation of the 40 KDa substrate of PKC by PAF vehicle (track 1), 0.3, 3, 30 and 300 nM PAF respectively (tracks 2-5) and with 100 nM TPA (track 6). Platelet activation has been terminated at 30s post-PAF addition. The phosphorylated PKC substrate band has been excised and subject to scintillation counting, and the results are given in (b) as a percentage of phosphorylation with PAF vehicle.

3.2.3 Protein kinase C and PAF-induced platelet activation

To explore the role of endogenous activators of PKC on the regulation of PAF-induced formation of Ins(1,4,5)P_3, DAG, TxB_2 and changes in Ca^{++} homeostasis, the effect of the potent PKC inhibitor staurosporine on these parameters was examined: 1 μM staurosporine was used as this concentration inhibits PKC activation by at least 70% (Fig 13).

3.2.3.1 Ins(1,4,5)P_3 and DAG

Pretreatment of the platelets with 1 μM staurosporine did not modify the basal concentrations of either Ins(1,4,5)P_3 or DAG (Fig 30). Thus PKC does not exert a tonic control over these parameters in the unstimulated platelets. The effect of staurosporine on PAF-stimulated formation of Ins(1,4,5)P_3 and DAG was dependent upon the concentration of PAF employed. Staurosporine produced a two and three fold enhancement of Ins(1,4,5)P_3 and DAG formation respectively in response to 300 nM PAF (Fig 30). When 3 nM PAF was used, which alone failed to produce an increase of Ins(1,4,5)P_3 or DAG over the basal levels, pretreatment with staurosporine did not produce any detectable increase over this.

Pretreatment of the platelets with the PKC activator TPA (100 nM) produced a significant reduction in PAF-stimulated levels of Ins(1,4,5)P_3 and DAG (Fig 30).

The effect of 1 μM staurosporine on 300 nM PAF-induced Ins(1,4,5)P_3 elevation over a 60 second time course demonstrates the transient elevation of Ins(1,4,5)P_3 both in the presence and absence of staurosporine (Fig 31a). Although at different levels, maximal elevation of Ins(1,4,5)P_3 has been reached by 5 s both in staurosporine treated or untreated platelets after which levels of Ins(1,4,5)P_3 decline at similar rates towards basal. This may indicate that PKC is exerting an effect on the production of Ins(1,4,5)P_3 rather than its metabolism (Fig 31a).

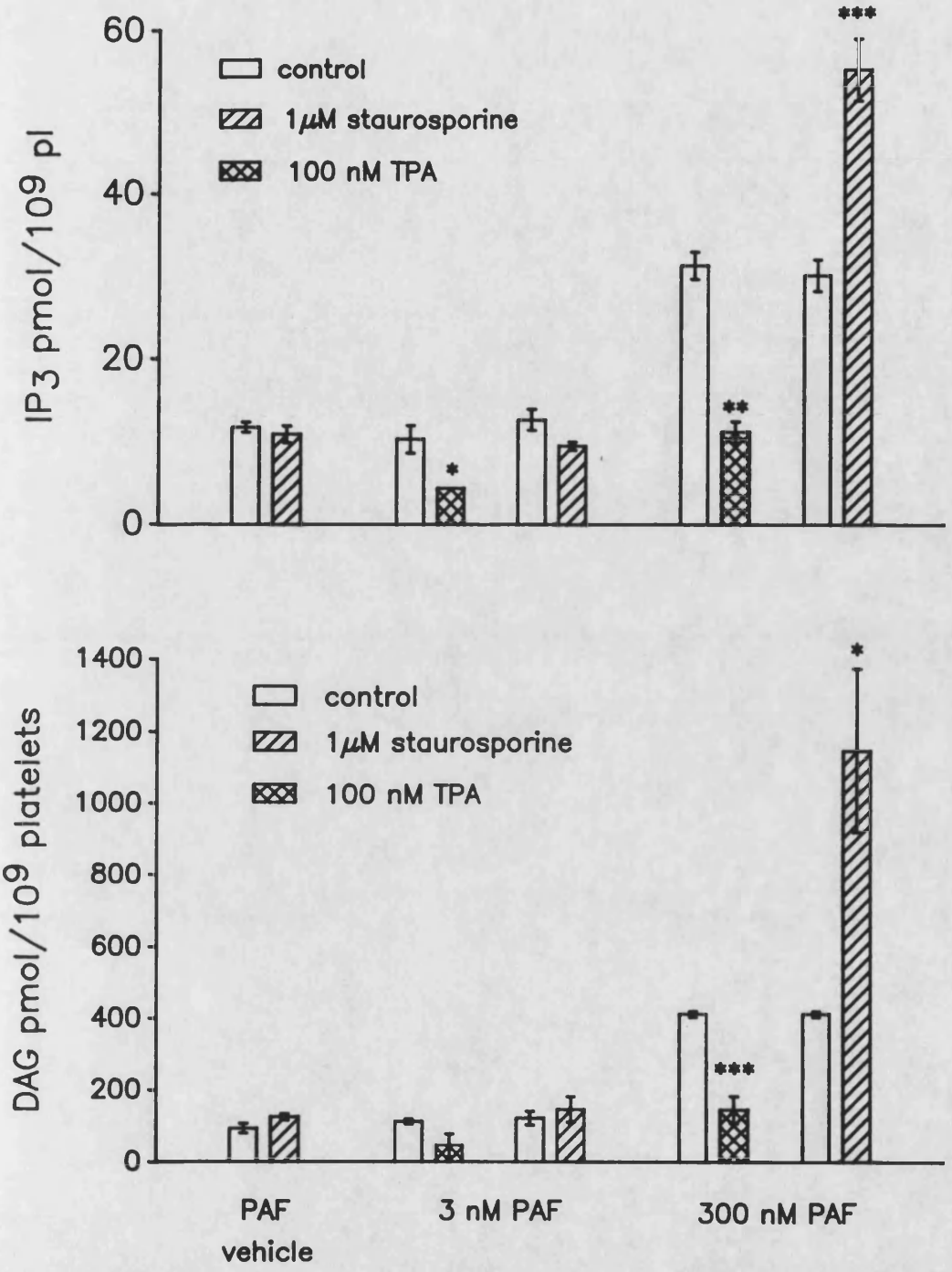


Fig 30 Effect of PKC activation or inhibition on generation of DAG and Ins(1,4,5)P_3 induced by 3 nM PAF or 300 nM PAF. PKC was activated with 100 nM 12-0-tetradecanylphorbol 13-acetate or inhibited with 1 μM staurosporine for two minutes prior to stimulation with PAF. DAG incubations (b) were terminated 10s after the addition of PAF, whilst Ins(1,4,5)P_3 incubations (a) were terminated at 5s. Each value represents the mean ± S.E.M. of at least three experiments each performed in duplicate.

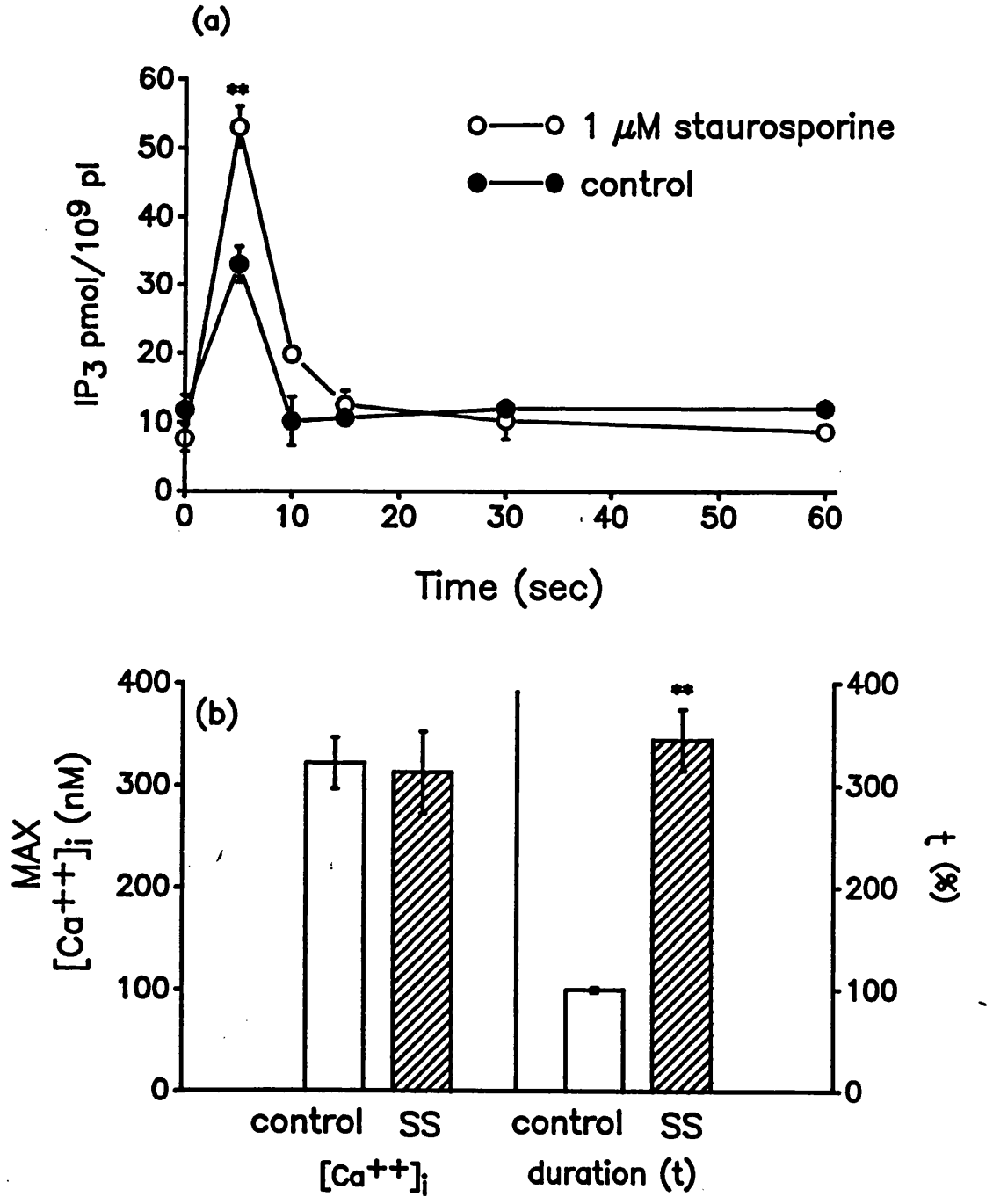


Fig 31 (a) Effect of staurosporine on PAF-induced Ins(1,4,5)P_3 elevation over a 1 minute time course (b) Effect of staurosporine on the maximal height and duration of decline of $[Ca^{++}]_i$ in platelets in the absence of $[Ca^{++}]_e$. Platelets were pretreated with either 1 μM staurosporine (open circle) or staurosporine vehicle (closed circle) for two minutes prior to activation with 300 nM PAF. Ins(1,4,5)P_3 production was terminated at predetermined times over a 1 minute time course. On the left of panel (b) $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-PAF value from the peak post-PAF value and on the right the duration of the decline of $[Ca^{++}]_i$ to basal levels (t) is given as a percentage of the control. Each value represents the mean ± S.E.M. of four experiments each performed in duplicate.

3.2.3.2 Calcium homeostasis

Pretreatment of platelets with 1 μM staurosporine did not modify the basal $[Ca^{++}]_i$ of platelets. In addition, pretreatment of platelets with staurosporine also had no significant effect on the rate of elevation (not shown) or the maximum elevation of $[Ca^{++}]i$, stimulated with either 3 or 300 nM PAF (Fig 32).

Conversely, the duration of the Ca^{++} signal, measured as the time taken to return to ½ maximum elevation above basal ($t_{1/2}$), was significantly potentiated by staurosporine for both concentrations of PAF (Fig 32). However, the increase in $t_{1/2}$ in platelets stimulated with 3nM PAF was only 30% in contrast to the 200% increase in $t_{1/2}$ observed in 300 nM stimulated platelets. Moreover, in platelets where the extracellular Ca^{++} had been replaced by EGTA the $T_{1/2}$ was also significantly potentiated in platelets stimulated with 300 nM PAF (Fig 31b).

At this point it is important to consider the effects of staurosporine on PAF-induced platelet aggregation. Pretreatment of platelets with staurosporine (60 - 2000 nM) caused a dose dependent inhibition of PAF-induced aggregation, although it did not affect PAF-induced platelet shape change. At a concentration of 2 μM, staurosporine inhibited PAF-induced platelet aggregation by 60%, and it had an IC_{50} of 1.75 μM (Fig 33a). It is therefore possible that the shorter duration of $[Ca^{++}]_i$ elevation observed in platelets not pretreated with staurosporine may be due to the presence of platelet aggregants, which could possibly interfere with and reduce the fluorescence signal and therefore the calculated duration of $[Ca^{++}]_i$ elevation. Conversely in staurosporine pretreated platelets removal of the interference of platelet aggregants would therefore increase the duration of the fluorescence signal. To remove this factor, the duration of $[Ca^{++}]_i$ was investigated in platelets which were prevented from aggregating. Prevention of platelet aggregation was effected either by terminating platelet stirring 10 s after the addition of PAF or pretreating platelets with 870 μM of the tetrapeptide ARG GLY ASP SER (Fig 33b). This short peptide sequence (termed RGDS) inhibits cellular attachment to fibrinogen and fibronectin to the platelet GPIIb/IIIa complex, thereby inhibiting platelet aggregation (Plow *et al.* 1985).

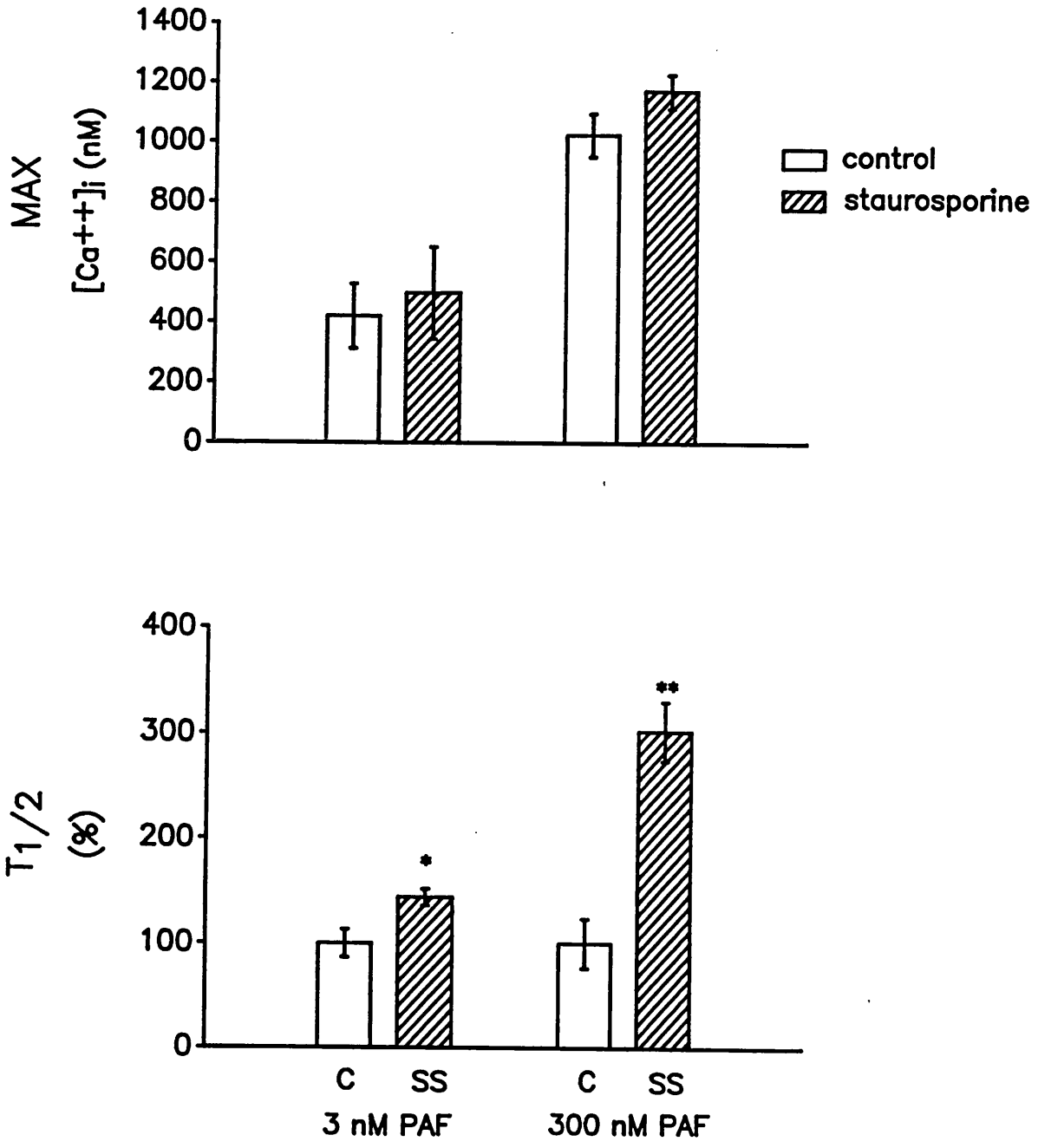


Fig 32 Effect of staurosporine on PAF-induced $[Ca^{++}]_i$ elevation. Effect of 1 μM staurosporine on the peak elevation of $[Ca^{++}]_i$ is shown in the upper panel and the effect of 1 μM staurosporine on the $t_{1/2}$ (time for decay of elevated $[Ca^{++}]_i$ to half maximal elevation over basal) is given in the lower panel, both in platelets stimulated with 3 nM or 300 nM PAF. $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-PAF value from the peak post-PAF value. Each value represents the mean ± S.E.M. of at least 3 experiments each performed in triplicate.

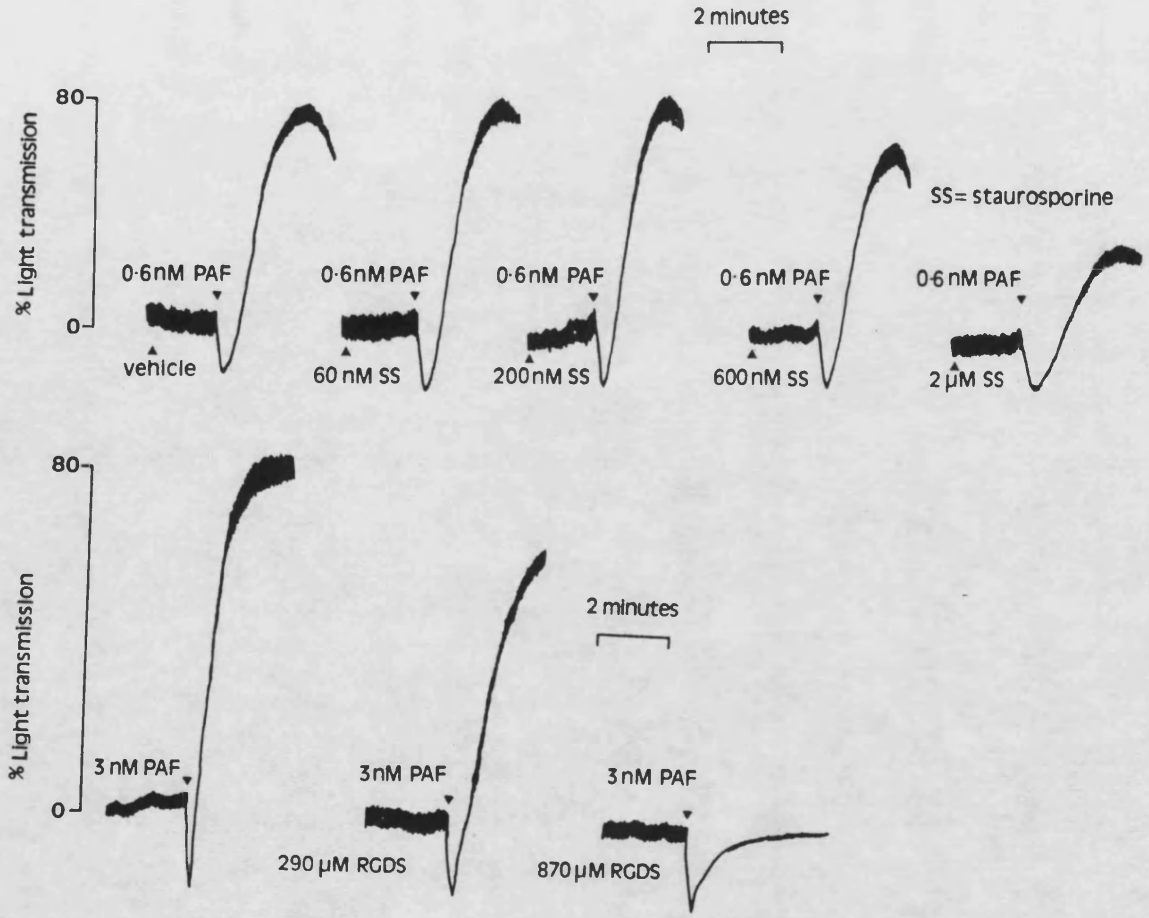


Fig 33 Effect of staurosporine or the tetrapeptide ARG GLY ASP SER on PAF-induced platelet aggregation. Upper panel, platelets were preincubated with staurosporine for two minutes prior to the addition of 0.6 nM PAF. Lower panel, platelets were preincubated with the tetrapeptide for two minutes prior to the addition of 3 nM PAF. Traces are taken from single experiments which are representative of two other similar experiments.

Inhibition of platelet aggregation either by preventing stirring or addition of RGDS had no effect on the duration of PAF-induced $[Ca^{++}]_i$ in platelets either pretreated with staurosporine or staurosporine vehicle (Fig 34).

3.2.3.3 Thromboxane B_2 formation

Addition of 300 nM PAF induced a rapid generation of TxB_2 with maximal rate of production over the first 10 s after platelet stimulation and with almost 80 percent of total release by 20 s post-PAF. At 2 minutes after PAF stimulation there was no further release of TxB_2 (Fig 35a). Only trace amounts of TxB_2 (<1 pmol/ml) were detected after platelet activation with either 0.3 or 3nM PAF, and these levels were not affected by staurosporine (Fig 35b). However, at higher concentrations of PAF (30-300nM) there was a dose dependent increase in the TxB_2 generated and this was potentiated at least 2-fold with staurosporine (Fig 35b).

To determine whether the staurosporine-enhanced TxA_2 formation was responsible for (a) the potentiation in the duration of elevated $[Ca^{++}]_i$ (see Fig 32) or (b) the increased formation of DAG (see Fig 30), the effect of indomethacin on these systems was examined (Fig 36). Pretreatment of the platelets with 2.8 μM indomethacin, which caused >99% inhibition of TxB_2 formation (see Fig 9), had no significant ($p>0.05$) effect on either basal levels of $[Ca^{++}]_i$ (Fig 36a) or DAG (Fig 36b & c). PAF-induced or staurosporine-enhanced duration of the $[Ca^{++}]_i$ signal (Fig 36a) or elevation of DAG (Fig 36b & c) was also unaffected by indomethacin pretreatment.

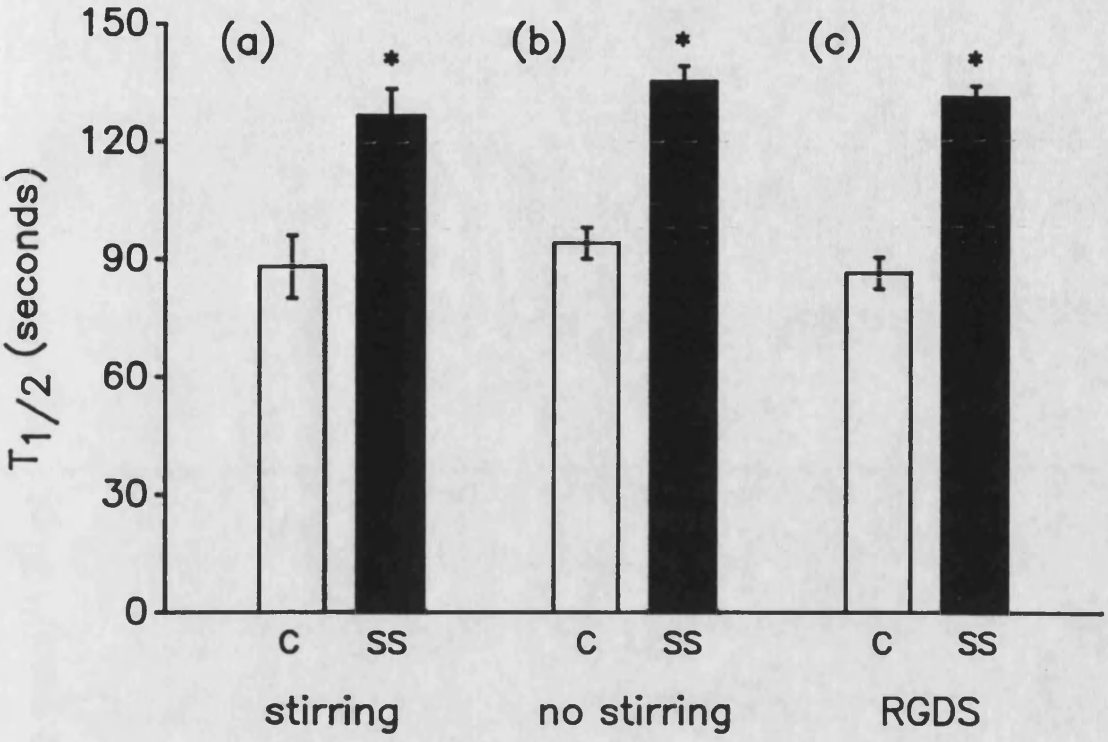


Fig 34 Effect of inhibiting platelet aggregation on the rate of decline of $[Ca^{++}]_i$ in PAF-stimulated platelets either in the presence or absence of staurosporine. Platelet aggregation was either not prevented (a) or was prevented by either stopping platelet stirring 10 s after PAF addition (b) or by the addition of 870 μM ARG GLY ASP SER (c). Platelets were pretreated with 1 μM staurosporine or staurosporine vehicle two minutes prior to platelet activation with 3 nM PAF. The values are the means ± S.E.M. of three separate experiments each carried out in triplicate.

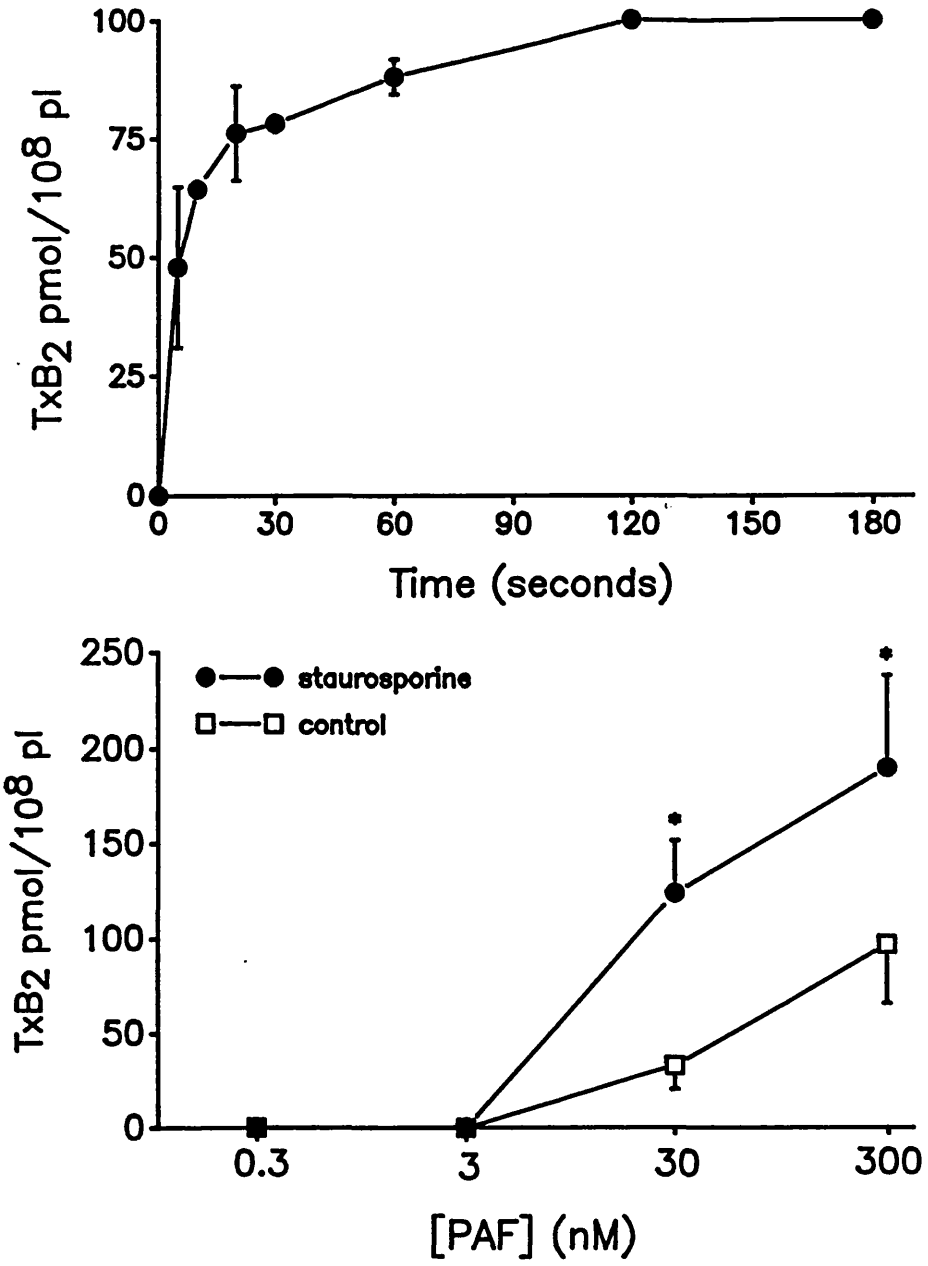
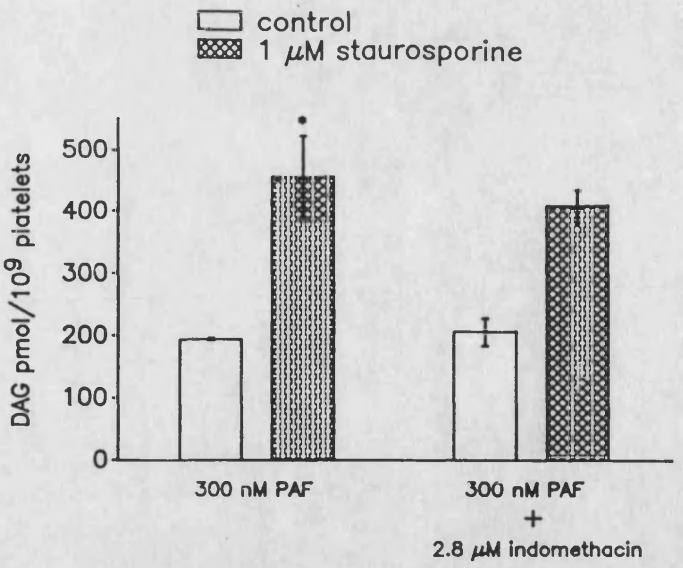
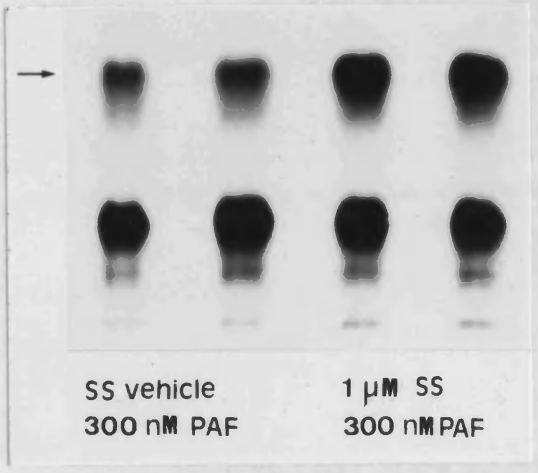
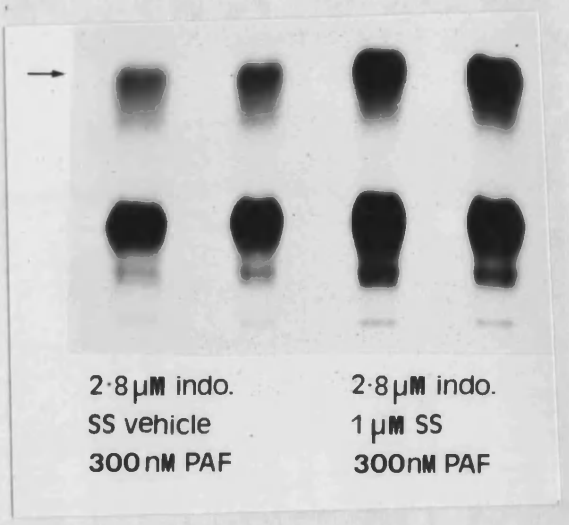


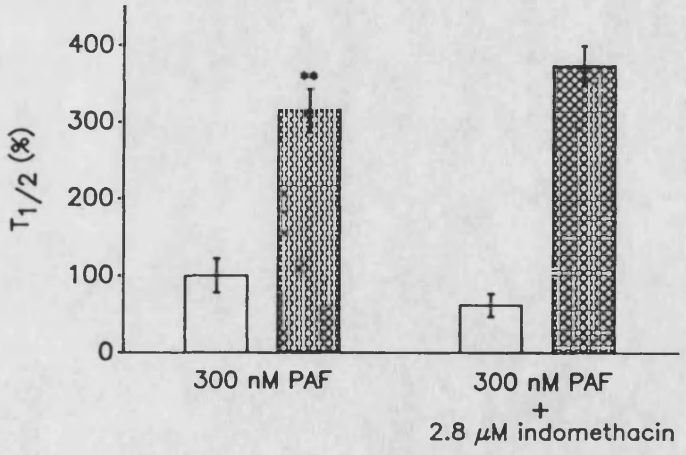
Fig 35 Time course of TxB_2 generation and the effect of 1 μM staurosporine on PAF-induced TxB_2 generation. (a) TxB_2 generation induced by 300 nM PAF over a three minute time course. (b) The effect of a 2 minute pre-incubation with staurosporine on TxB_2 generation induced PAF (0.3, 3, 30 & 300 nM) was determined at 3 minutes post-PAF. The points represent the mean $\pm$ S.E.M. of 3 experiments each performed in triplicate.

Fig 36 The effect of 2.8 μM indomethacin on DAG generation and the $t_{1/2}$ of $[Ca^{++}]_i$ in the presence and absence of staurosporine. Platelets either in the presence or absence of 2.8 μM indomethacin were pretreated with either 1 μM staurosporine or staurosporine vehicle for two minutes prior to stimulation with 300 nM PAF. Upper panel, the effect of indomethacin on PAF-induced DAG elevation, measured 10 s post-PAF stimulation. Middle panel, autoradiographies demonstrating the effect of 2.8 μM indomethacin on PAF-induced DAG production (after conversion to [^{32}]P-Phosphatidic acid) either in the presence or absence of staurosporine. Lower panel, the effect of indomethacin on the $t_{1/2}$ of $[Ca^{++}]_i$ in PAF-stimulated platelets either in the presence or absence of 1 μM staurosporine. The values represent the mean $\pm$ S.E.M. of at least three experiments each performed in triplicate.


control
1 μM staurosporine
DAG pmol/10⁹ platelets
500
400
300
200
100
0
300 nM PAF
300 nM PAF
+
2.8 μM indomethacin


SS vehicle
300 nM PAF
1 μM SS
300 nM PAF


2·8 μM indo.
SS vehicle
300 nM PAF
2·8 μM indo.
1 μM SS
300 nM PAF


T1/2 (%)
400
300
200
100
0
300 nM PAF
300 nM PAF
+
2.8 μM indomethacin

3.3 Investigation of the role of endogenously activated PKC in platelet functional responses using selective inhibitors of PKC.

Staurosporine has been widely used to investigate the role of PKC in many different cell types. However, although it is a potent protein kinase inhibitor it is not selective for PKC. During the course of this study inhibitors of PKC which are both potent and selective were developed. Therefore, it was important to look at the effects of these inhibitors on platelet functional responses in order to assess the role of endogenously activated PKC in platelets where other kinases were not being inhibited and also to compare the results with the findings we and others have obtained when using staurosporine. The structures of the four PKC inhibitors staurosporine (Tamaoki *et al.*1986), calphostin C (Kobayashi *et al.*1989) and Roche 31-7549/001 and 31-8220/002 (Davis *et al.*1989) are given in Fig 37.

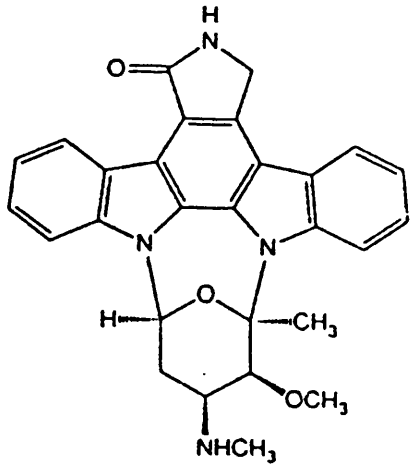
3.3.1 Calphostin C

A 20 minute preincubation of platelets with the selective PKC inhibitor calphostin C inhibited 300 nM PAF-induced phosphorylation of the 40-47 kDa major PKC substrate dose dependently with an IC_{50} of 1 μM (Fig 38).

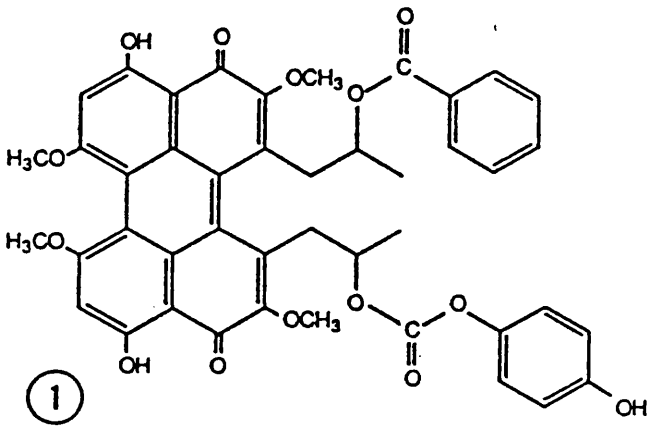
In contrast to staurosporine (see Figs 14 & 16) calphostin C caused a dose dependent inhibition of both $[Ca^{++}]_i$ elevation and TxB_2 generation in platelets stimulated with PAF (Fig 38). However, in common with staurosporine (see Fig 23b), calphostin C inhibited 5-HT release in PAF-stimulated platelets (Fig 39). Calphostin C caused inhibition of both ionomycin-induced TxB_2 generation and 5-HT release similar to the results obtained with staurosporine in ionomycin stimulated platelets (Fig 40).

3.3.2 Ro 31-7549/001 and Ro 31-8220/002

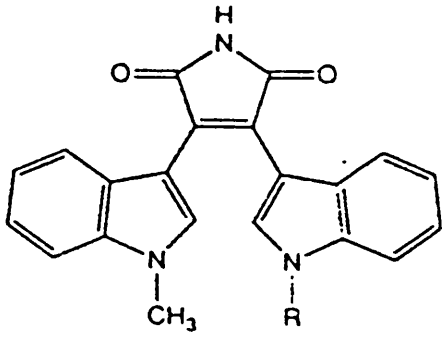
A 20 minute pre-incubation with the selective PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002 inhibited 300 nM PAF-induced phosphorylation of the 40-47 KDa major PKC substrate in a dose dependent manner (Fig 41). Ro 31-7549/001 inhibited phosphorylation with an IC_{50} of 2.5 μM whilst Ro 31-8220/002 inhibited phosphorylation with an IC_{50} of 0.45 μM.



Staurosporine



Calphostin C



Roche Compounds

Ro 31-7549/001 R = $(CH_2)_3NH_2$

Ro 31-8220/002 R = $(CH_2)SC(=NH)NH_2$

Fig 37 The structures of the 4 PKC inhibitors staurosporine, calphostin C, Ro 31-7549/001 and Ro 31-8220/002.

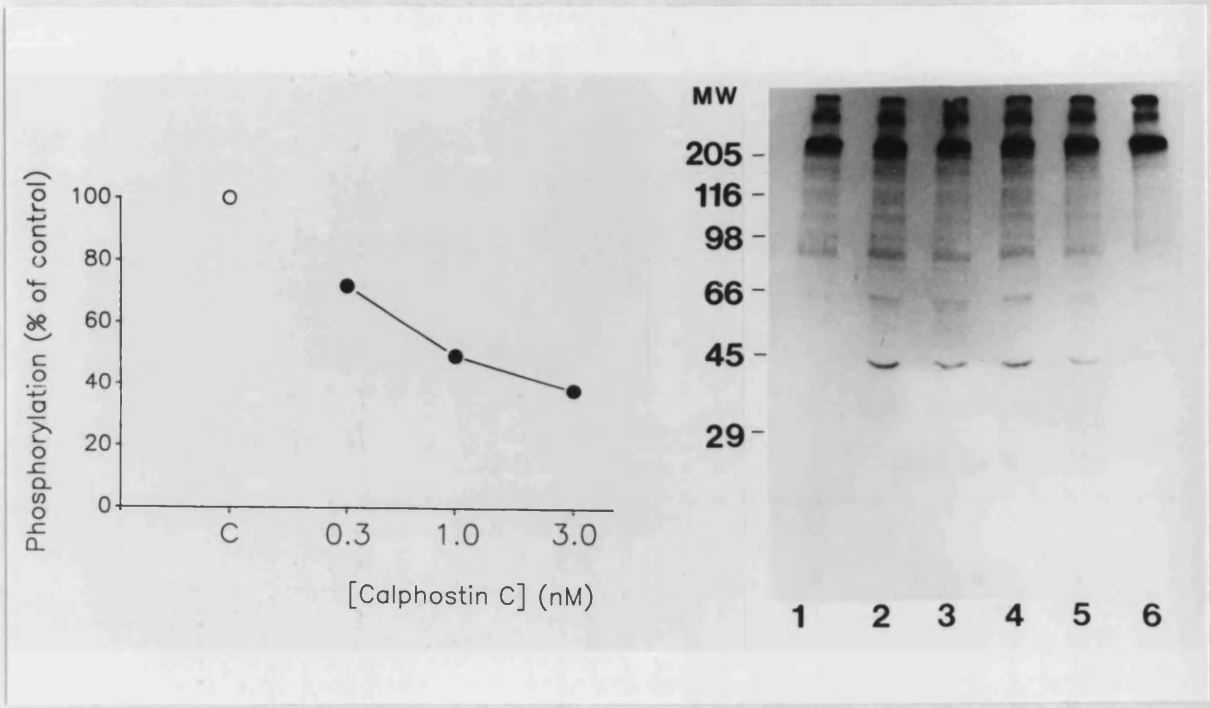


Fig 38 The effect of calphostin C on PAF-induced phosphorylation of the 40 kDa substrate of PKC. (Right panel) Autoradiograph showing phosphorylation of platelet proteins. Track 1, resting platelets; track 2, 300 nM PAF stimulated platelets; tracks 3-6 respectively, platelets pretreated for 20 minutes with 100, 300, 1000 and 3000 nM calphostin C and then stimulated with 300 nM PAF. The phosphorylated PKC substrate was excised and subjected to scintillation counting and the results are given in the left panel as a percentage of the phosphorylation in the presence of 300 nM PAF. The autoradiography shown is representative of four individual experiments and the data are the mean values taken from the four experiments.

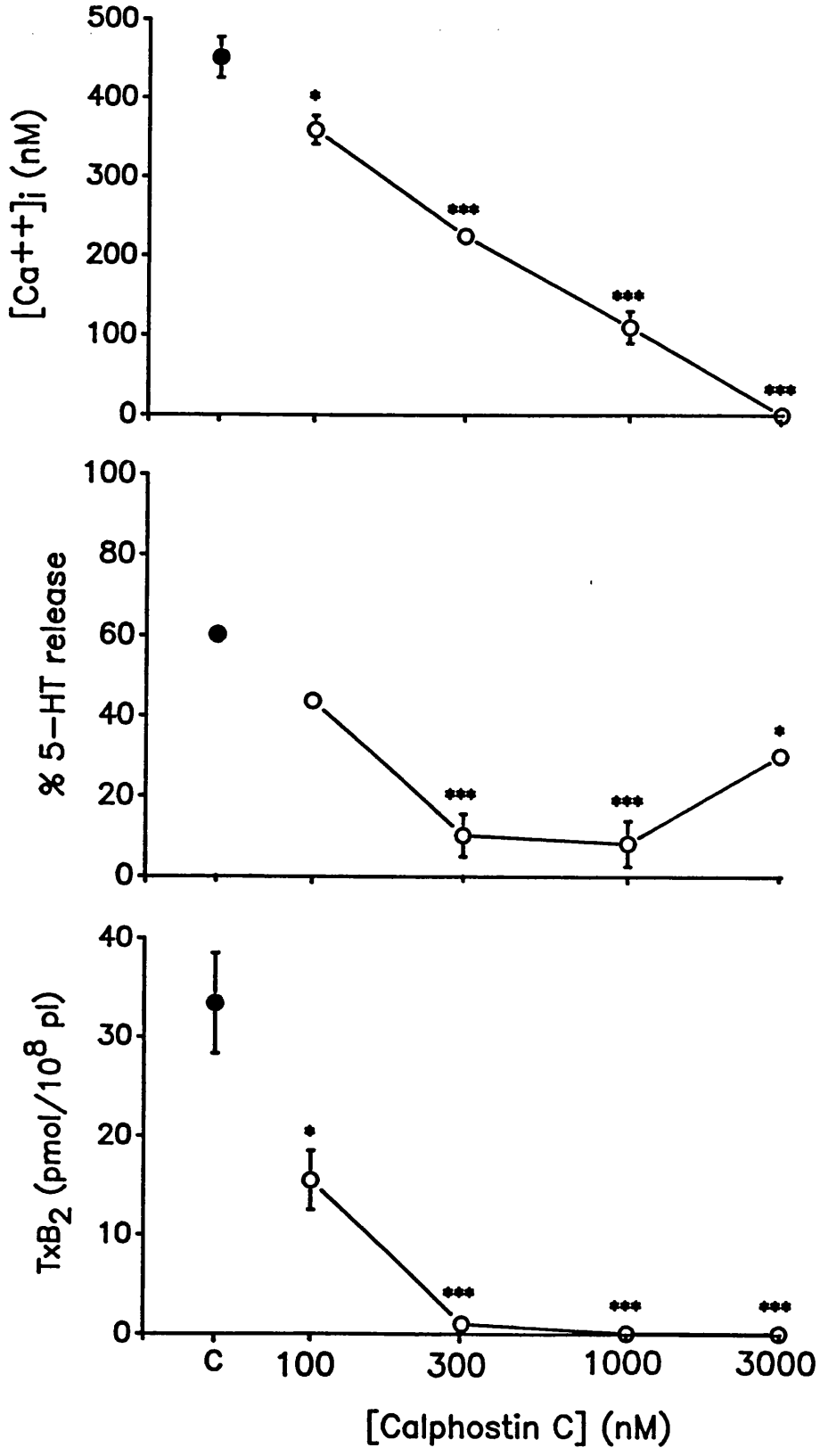


Fig 39 The effect of calphostin C on PAF-induced $[Ca^{++}]_i$ elevation, 5-HT release and TxB_2 generation. Platelets were preincubated with calphostin C for 20 minutes prior to PAF stimulation. $[Ca^{++}]_i$ elevation (upper panel) is the peak elevation induced by 3 nM PAF after subtracting the basal pre-PAF value. 5-HT release induced by 3 nM PAF (middle panel) and TxB_2 generation induced by 30 nM PAF (lower panel) were terminated 3 minutes after the addition of PAF. Each of the values is the mean ± S.E.M. of 3 experiments each performed in triplicate.

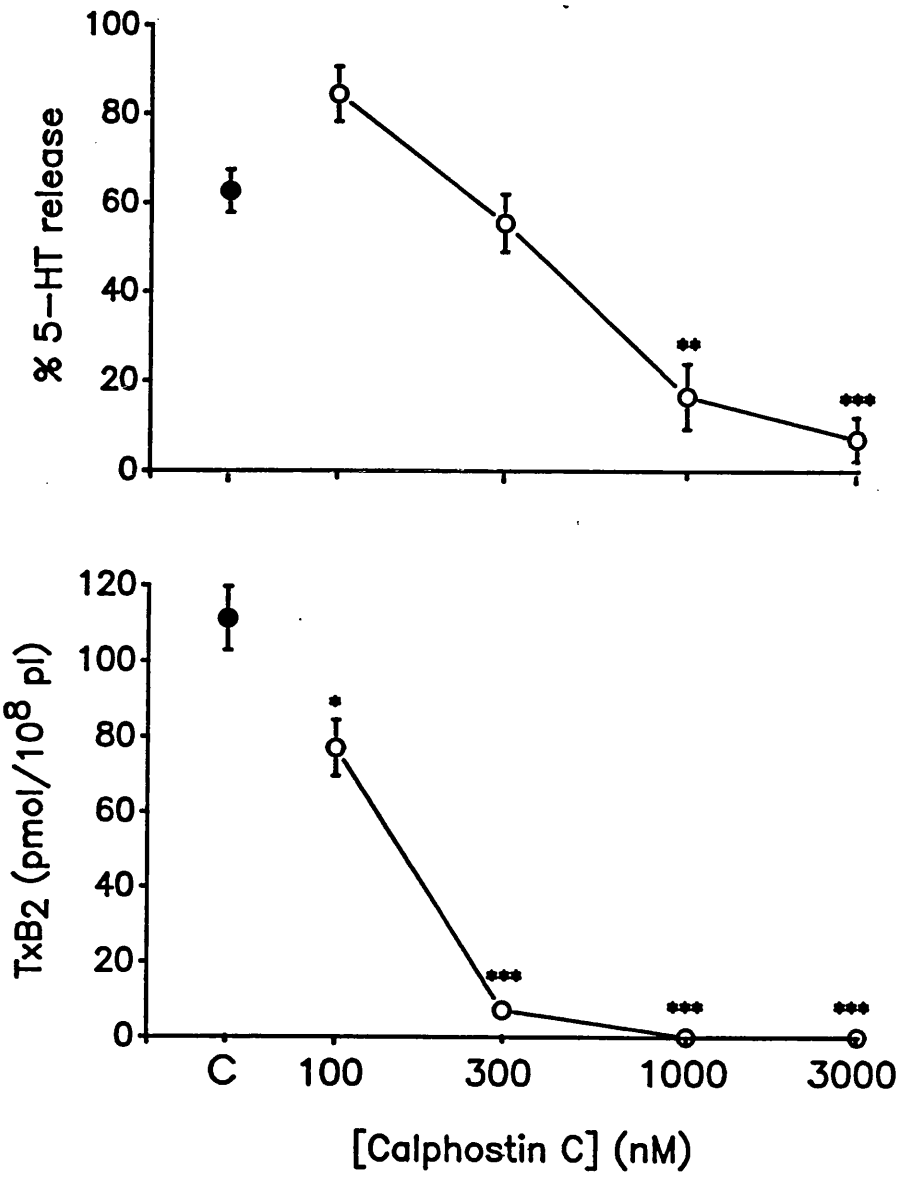


Fig 40 The effect of calphostin C on 5 μM ionomycin-induced 5-HT release and TxB_2 generation. Platelets were pre-treated with calphostin C for 20 minutes before stimulation with 5 μM ionomycin. 5-HT release (upper panel) and TxB_2 generation (lower panel) were terminated 3 minutes after ionomycin-induced activation. The values are the ± S.E.M. of three separate experiments each performed in triplicate.

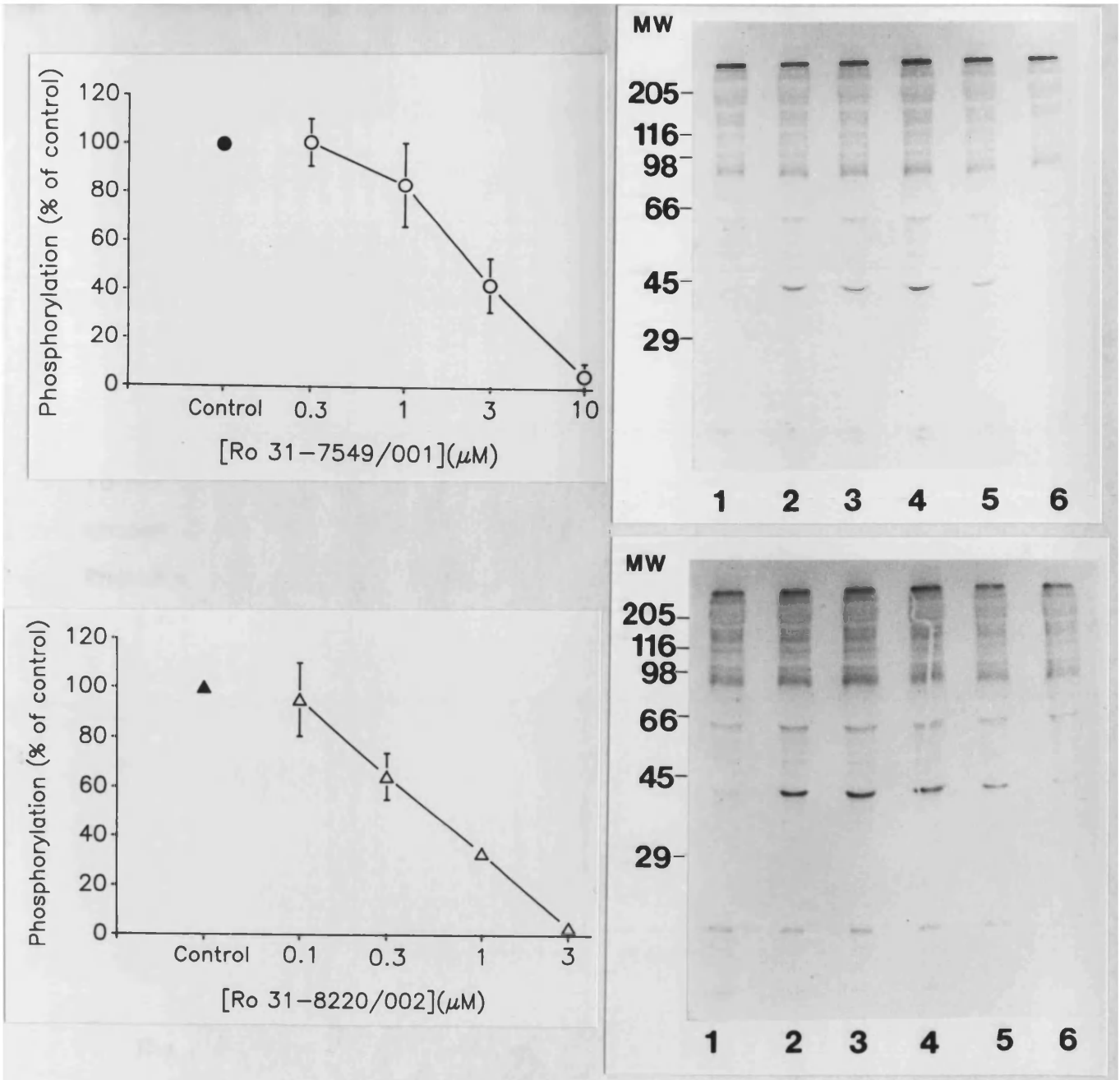


Fig 41 Inhibition of PAF-induced phosphorylation of the 40-47 kDa substrate of PKC by Ro 31-7549/001 (upper panel) and Ro 31-8220/002 (lower panel). The right side of the panels are autoradiographs representing phosphorylation in resting platelets (track 1), 300 nM PAF-induced platelet phosphorylation in the presence of the respective PKC inhibitor vehicle (track 2), and a 20 minute pre-incubation with either 0.3,1,3,10 μM Ro 31-7549/001 (upper panel) or 0.1,0.3,1,3 μM Ro 31-8220/002 (lower panel) followed by 300 nM PAF stimulation for 30 seconds (tracks 3-6 respectively). Phosphorylation was quantified by scintillation counting of the excised [^{32}P]P_i labelled substrate. The results are given on the left of each panel as a percentage of phosphorylation induced by 300 nM PAF. Each point is the mean ± S.E. of three separate experiments.

3.3.3 Effect of PKC inhibition by Ro 31-7549/001 and Ro 31-8220/002 on PAF-induced functional responses

3.3.3.1 $[Ca^{++}]_i$ elevation

The effect of inhibition of PKC on $[Ca^{++}]_i$ elevation was investigated using both submaximal (3 nM) and maximal (300 nM) PAF-stimulated platelets (Fig 42). 10 μM of Ro 31-7549-001 and 3 μM of Ro 31-8220-002 were used as these concentrations were found to inhibit PKC activation by >90% (see Fig 41). Pretreatment with either Ro 31-7549/001 or Ro 31-8220/002 for 20 minutes had no effect on maximal height of $[Ca^{++}]_i$ elevation induced by either 3 or 300 nM PAF however, both inhibitors significantly increased the duration of the Ca^{++} signal (Fig 42). Moreover, whereas in 300 nM PAF stimulated platelets inhibition of PKC caused 180% increase in duration of the Ca^{++} signal, it only caused about a 50% increase with 3 nM PAF (Fig 42). These findings were comparable to those found when using staurosporine to inhibit PKC (see Fig 32).

3.3.3.2 Reversal of TPA-induced PKC activation by using Ro 31-7549/001 and Ro 31-8220/002

The ability of the PKC inhibitors to abrogate the effect of PKC activation was studied by investigating their ability to reverse the effect of the PKC activator TPA on PAF induced $[Ca^{++}]_i$ elevation (Fig 43). Activation of PKC with 30 nM TPA inhibited $[Ca^{++}]_i$ elevation (Fig 43) in PAF-stimulated platelets. A 20 minute pre-incubation with the PKC inhibitors completely abrogated the effect on $[Ca^{++}]_i$ caused by TPA.

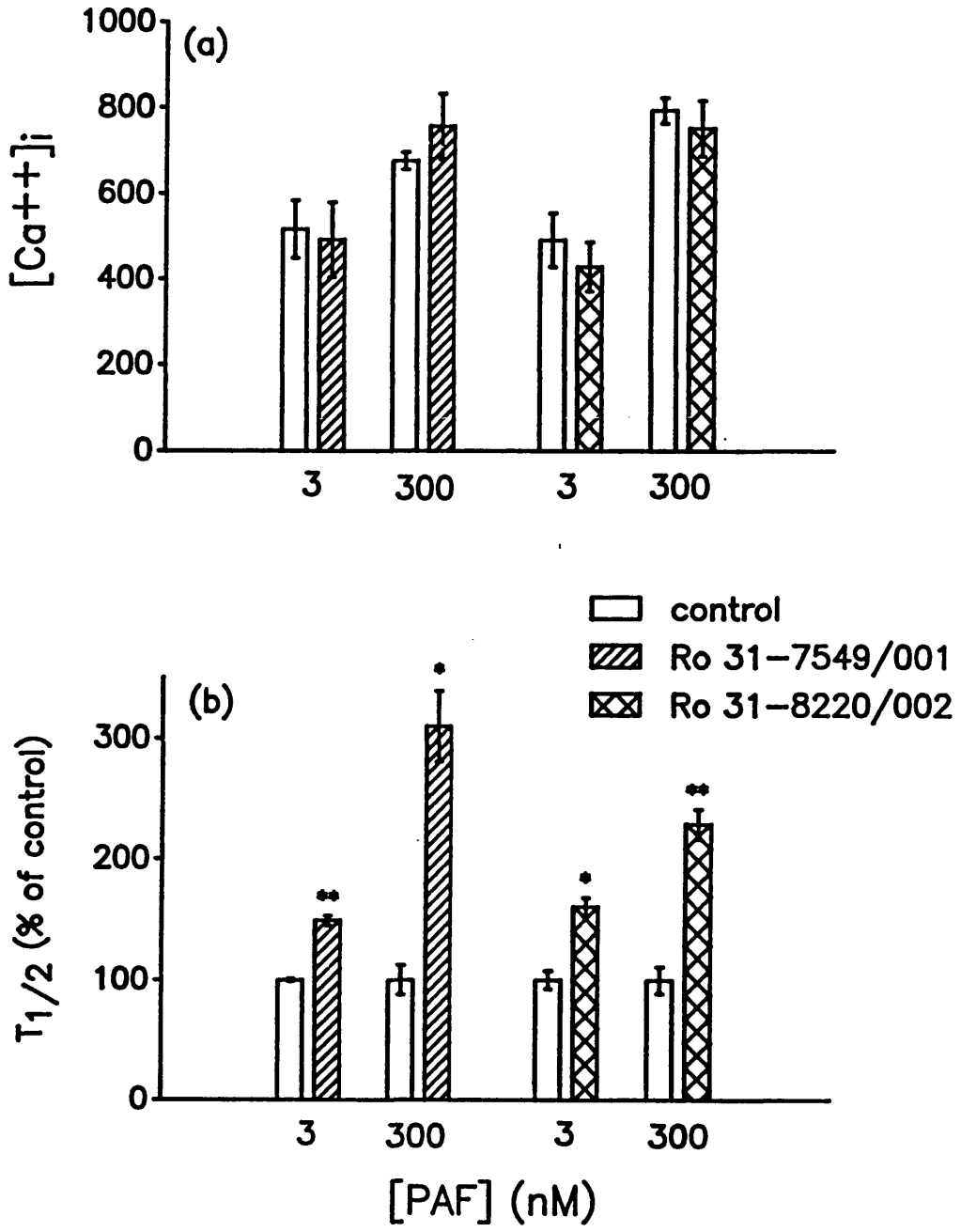


Fig 42 Effect of the Roche compounds on maximal $[Ca^{++}]_i$ elevation and on the duration of $[Ca^{++}]_i$ elevation in 3 and 300 nM PAF stimulation platelets. Platelets were incubated with either 10 μM Ro 31-7549/001 or 3 μM Ro 31-8220/002 or their appropriate vehicle (<0.1% DMSO) 20 minutes prior to the addition of PAF. $[Ca^{++}]_i$ is the maximal elevation in cytosolic calcium after subtracting the basal pre-PAF value. $T_{1/2}$ is the time for calcium to decline to 1/2 maximal above basal levels. Results are expressed as a % of the control $t_{1/2}$. The values are the means ± S.E.M. of three separate experiments each performed in triplicate.

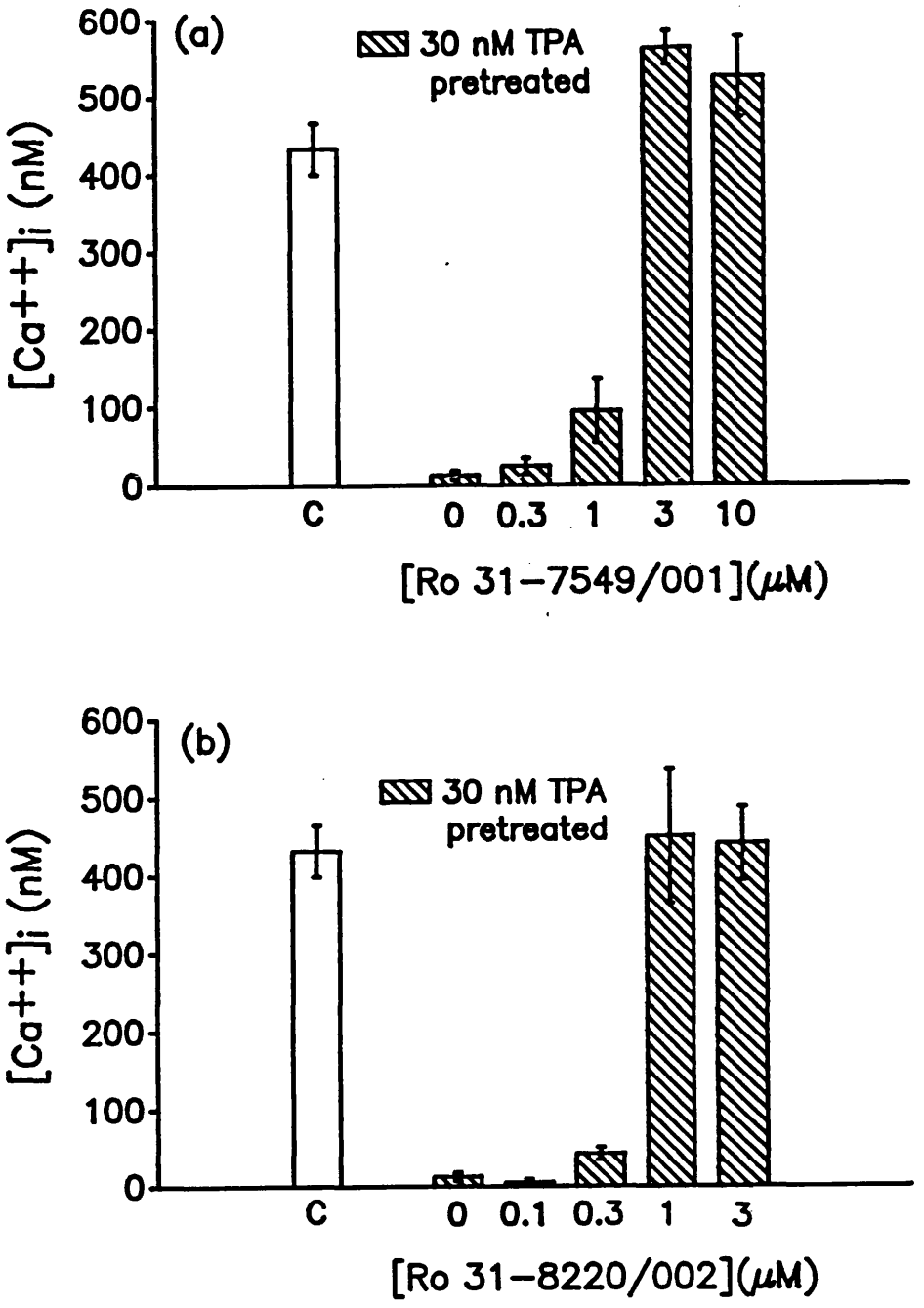


Fig 43 Abrogation of TPA-induced inhibition of $[Ca^{++}]_i$ elevation by Ro 31-7549/001 (a) or Ro 31-8220/002 (b). Platelets were pretreated with the PKC inhibitors or vehicle 20 minutes before addition of the PKC activator TPA (30 nM) which was incubated for 2 minutes before stimulation with 3 nM PAF. $[Ca^{++}]_i$ is the value obtained by subtracting the basal pre-PAF value from the maximal post-PAF value. Data is from 3 separate experiments each performed in triplicate.

3.3.3.3 TxB_2 generation

Using 10 μM Ro 31-7549/001 and 3 μM Ro 31-8220/002 (concentrations which inhibited PKC substrate phosphorylation by >90%) the effect of inhibition of PKC on TxB_2 generation was investigated in platelets stimulated with 30 nM PAF (Fig 44). Both PKC inhibitors caused a 3 - 5 fold potentiation of TxB_2 generation induced by 30 nM PAF. Similarly to the results for $[Ca^{++}]_i$ elevation, inhibition of TxB_2 generation by TPA was abrogated by the PKC inhibitors (Fig 44). These findings indicate that the inhibition of $[Ca^{++}]_i$ elevation and TxB_2 generation by TPA in PAF stimulated platelets is mediated by PKC and can be reversed by pretreatment with either PKC inhibitor.

3.3.3.4 5-HT release

5-HT release induced by 3 nM PAF was dose-dependently inhibited when platelets were subjected to a 20 minute pretreatment with increasing concentrations of Ro 31-7549/001 (0.3,1,3,10 μM) or Ro 31-8220/002 (0.1,0.3,1,3 μM). The IC_{50} for Ro 31-7549/001 was 4.7 μM whilst for Ro 31-8220/002 it was 0.55 μM (Fig 45).

3.3.3.5 Aggregation

Aggregation of platelets induced by a submaximal concentration of PAF (1 nM) was dose-dependently inhibited with increasing concentrations of either PKC inhibitor, with almost total inhibition of platelet aggregation at 10 μM Ro 31-7549/001 or 3 μM Ro 31-8220/002. The IC_{50} for Ro 31-7549/001 was 1.8 μM whilst for Ro 31-8220/002 it was 0.47 μM (Fig 46). The PKC inhibitors did not however affect 1 nM PAF-induced shape change in platelets.

Table 4 is a summary of the effects of the different inhibitors of PKC, namely staurosporine, Ro 31/7549-001, Ro 31/8220-002 and calphostin C on PAF-induced PKC substrate phosphorylation, duration of $[Ca^{++}]_i$ elevation, TxB_2 generation and aggregation.

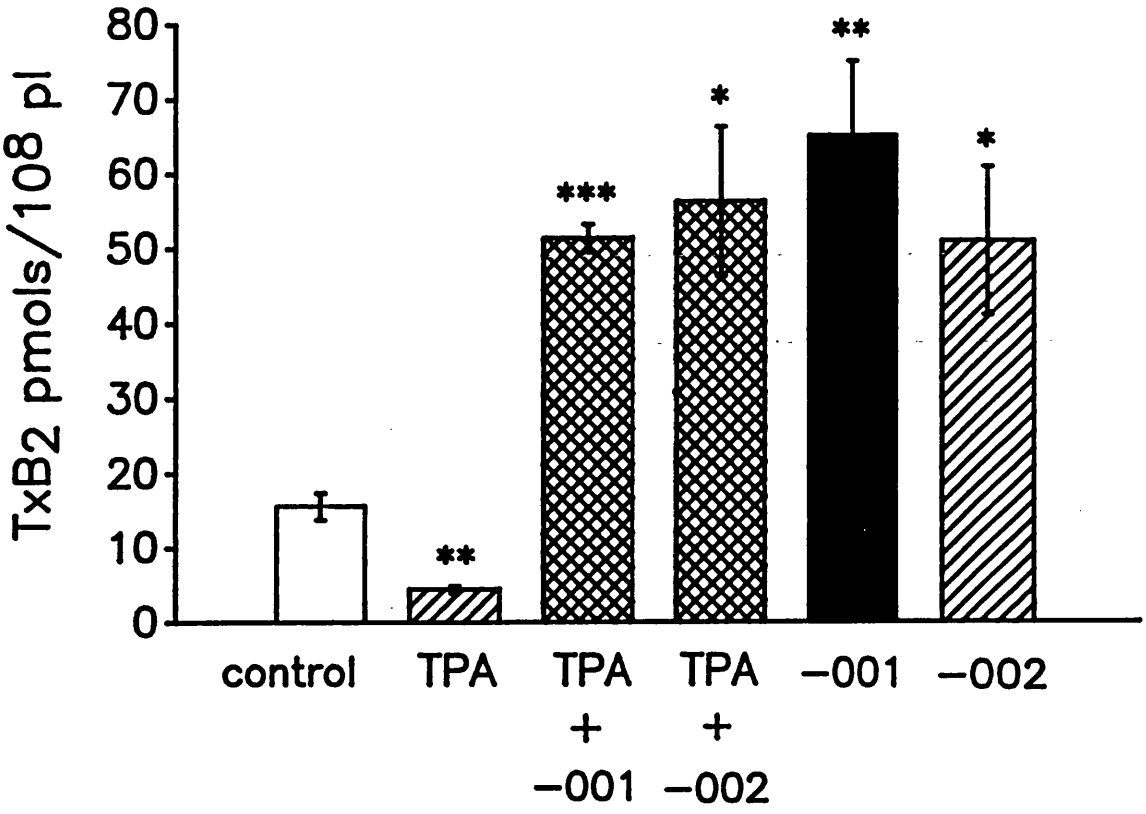


Fig 44 The effect of 10 μM Ro 31-7549/001 or 3 μM Ro 31-8220/002 on TxB_2 generation in 30 nM PAF-stimulated platelets and the abrogation of TPA-induced inhibition of TxB_2 generation by each of the PKC inhibitors. Platelets were pretreated with the PKC inhibitors or vehicle 20 minutes before the addition of the PKC activator TPA (30 nM) which was incubated for 2 minutes prior to stimulation with 30 nM PAF. TxB_2 generation was measured at 3 minutes post-PAF. Ro 31-7549/001 and Ro 31-8220/002 are abbreviated to -001 and -002 respectively in the figure. Control = platelets stimulated with 30 nM PAF. The results are the mean ± S.E.M of three separate experiments each performed in triplicate.

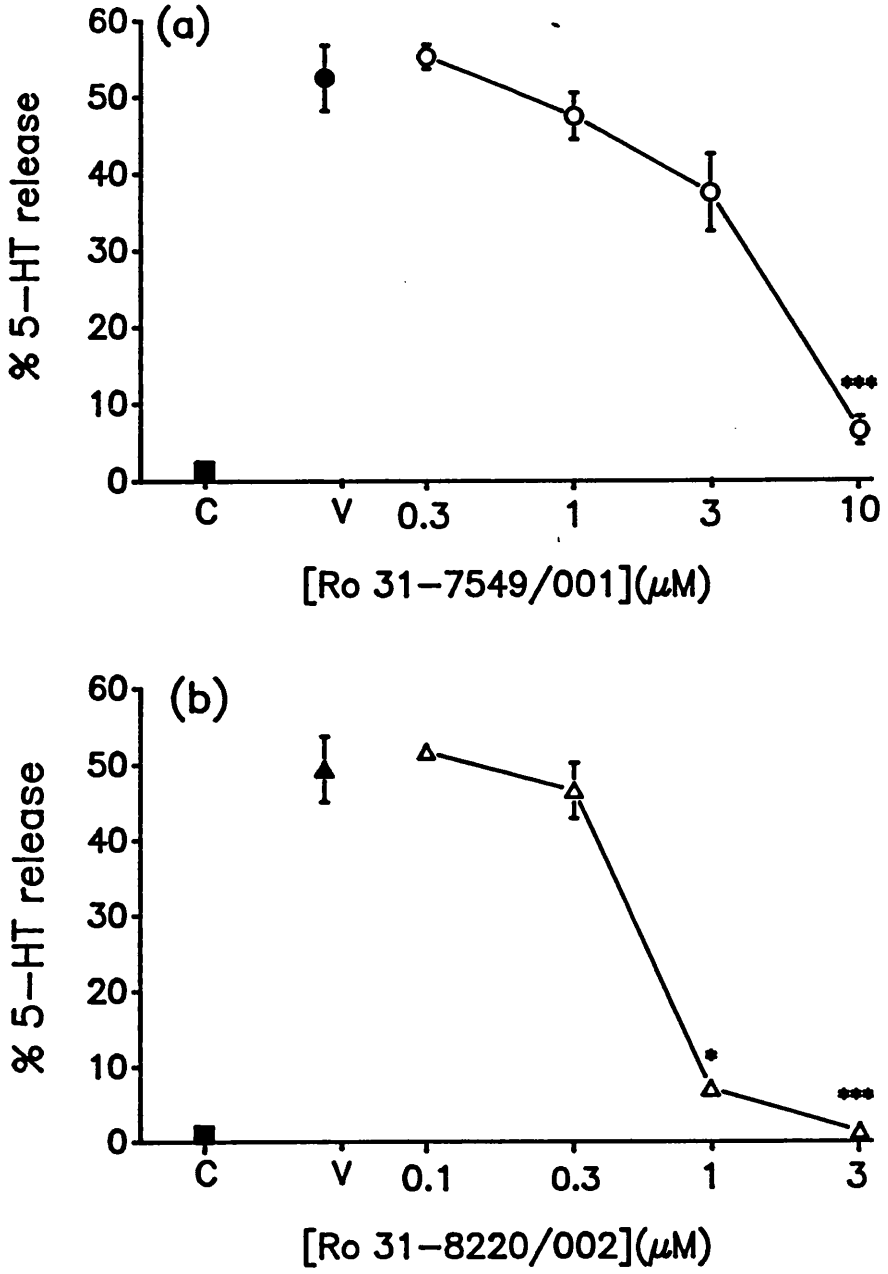


Fig 45 The effect of the PKC inhibitors on dense granule release induced by PAF. Platelets were treated for 20 minutes with Ro 31-7549/001 (upper panel) or Ro 31-8220/002 (lower panel) prior to activation with 3 nM PAF. The % 5-HT release induced by the highest concentration of each PKC inhibitor alone is shown as the closed square in each panel, whilst the effect of the PKC inhibitor vehicle on 3 nM PAF-induced granule release is shown as the closed circle and the closed triangle for Ro 31-7549/001 and Ro 31-8220/002 respectively. Dense granule release was terminated 3 minutes after the addition of PAF. The values are the mean ± S.E.M. of three separate experiments each performed in triplicate.

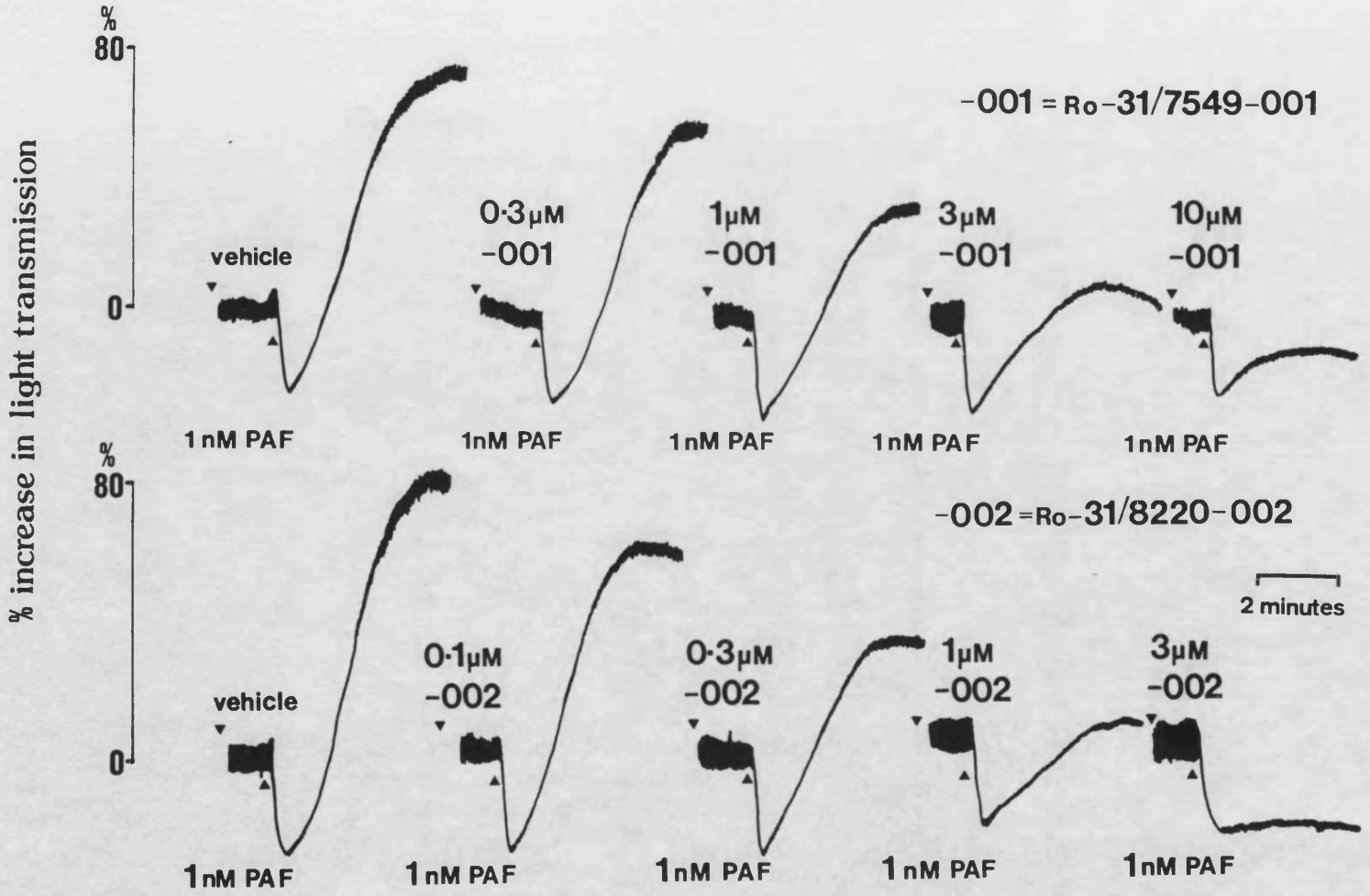


Fig 46 Traces showing the effect of PKC inhibition on PAF-induced platelet shape change and aggregation. Platelets were pretreated for 20 minutes with either Ro 31-7549/001 (top panel) or Ro 31-8220/002 (lower panel). The traces are taken from a single experiment but are representative of two other similar experiments.

	IC_{50} values 40 kDa substrate phosphorylation	fold increase in $[Ca^{++}]_i$ $T_{1/2}$	fold increase in TxB_2	aggregation
Staurosporine	0.5 μM	200 % *	200 % *(1 μM)	↓
Ro 31/7549-001	2.5 μM	200 % *	210 % *(10 μM)	↓
Ro 31/8220-001	0.45 μM	180 % *	300 % *(3 μM)	↓
Calphostin C	1.0 μM	↓	↓	-

* the concentration of inhibitor to give this fold increase in $[Ca^{++}]_i$ $T_{1/2}$ and TxB_2 generation.

Table 4 Summary of the effects of the four PKC inhibitors staurosporine, Ro 31/7549-001, Ro 31/8220-002 and calphostin C on PAF-induced PKC substrate phosphorylation, duration of $[Ca^{++}]_i$ and TxB_2 generation.

3.4 Development of the technique of Western Blotting

Several different techniques were explored in order to determine optimal conditions for Western blotting (see section 2.3.13.3). These included finding an optimal time for transferring protein from the SDS gel to the nitrocellulose, and optimal conditions for blocking the non-specific binding sites on the nitrocellulose. In order to determine the conditions which would give the clearest results for the Western blots, it was also necessary to find the best length of time for incubating with the primary and secondary antibodies and the optimal concentrations at which to use them. The washing procedure used to remove antibody which was not specifically bound to the Western blot was also investigated. In addition several methods reported to enhance detection of the antigen of interest were investigated, and it was found that using peroxidase anti-peroxidase antibody enhanced the 'signal' for the antigen. Having determined optimal conditions in which to perform Western blotting this technique was then used to determine the presence of a number of different annexins in platelets and also to investigate tyrosine kinase activity using an anti-phosphotyrosine antibody.

3.5 Identification and location of annexin isoforms in human platelets

The finding that inhibition of PKC with staurosporine caused potentiation of TxB_2 generation whilst PKC activation caused inhibition suggested that PKC may be one of the factors responsible for regulating PLA_2. It has previously been demonstrated that annexins are inhibitors of PLA_2 and it is possible that, if present in platelets, they may be controlled by protein kinases and be responsible for regulating PLA_2 activation. In this study the presence of annexins I,II,IV and V in human platelets was determined by Western blotting. Human platelets as opposed to rabbit platelets were used, as the antibodies available to annexins do not cross-react with rabbit.

3.5.1 Identification of the annexins present in platelets

The presence of annexins I,II,IV and V in platelets and U937 cells was determined by Western blotting (Fig 47). Using the polyclonal antibody against whole recombinant annexin I and against amino acids 1-188 of annexin I, it was identified as a 36 kDa protein in both platelets and U937 cells. Probing with antibodies against smaller sequences of annexin I (amino acids 24-37 and 38-50), however revealed higher molecular weight species of 70 kDa and 55 kDa respectively in platelets, whilst remaining at 36 kDa in U937 cells. The antibody to annexin II also revealed a higher molecular weight species of 68 kDa in platelets compared to U937 cells where annexin II had an apparent molecular weight of 35 kDa. Annexin IV was not detected in either total platelet lysates or U937 cell lysates, whilst annexin V appeared in both as a protein of 32 kDa molecular weight. Moreover in U937 cells (which had been pretreated with TPA see section 2.2.13.5.3) anti-annexin I antibody detected a doublet of 36 kDa and 32 kDa, whilst anti-annexin V antibody detected a doublet of 32 kDa and 29 kDa. In untreated platelets however the lower molecular band (of the doublet seen in U937 cells) was either missing or gave a much weaker signal than seen for U937 cells (Fig 47).

3.5.2 The location of annexins in platelets

In order to determine whether annexins I,II and V were located on the external surface of the plasma membrane, platelets were washed with EGTA which by chelating Ca^{++} should release the annexins since they bind to phospholipids in a Ca^{++} dependent manner. No annexin (I,II or V) was released by EGTA (Fig 48) suggesting that either they are not located on the outer surface of the plasma membrane, or that the membrane association is not Ca^{++} sensitive.

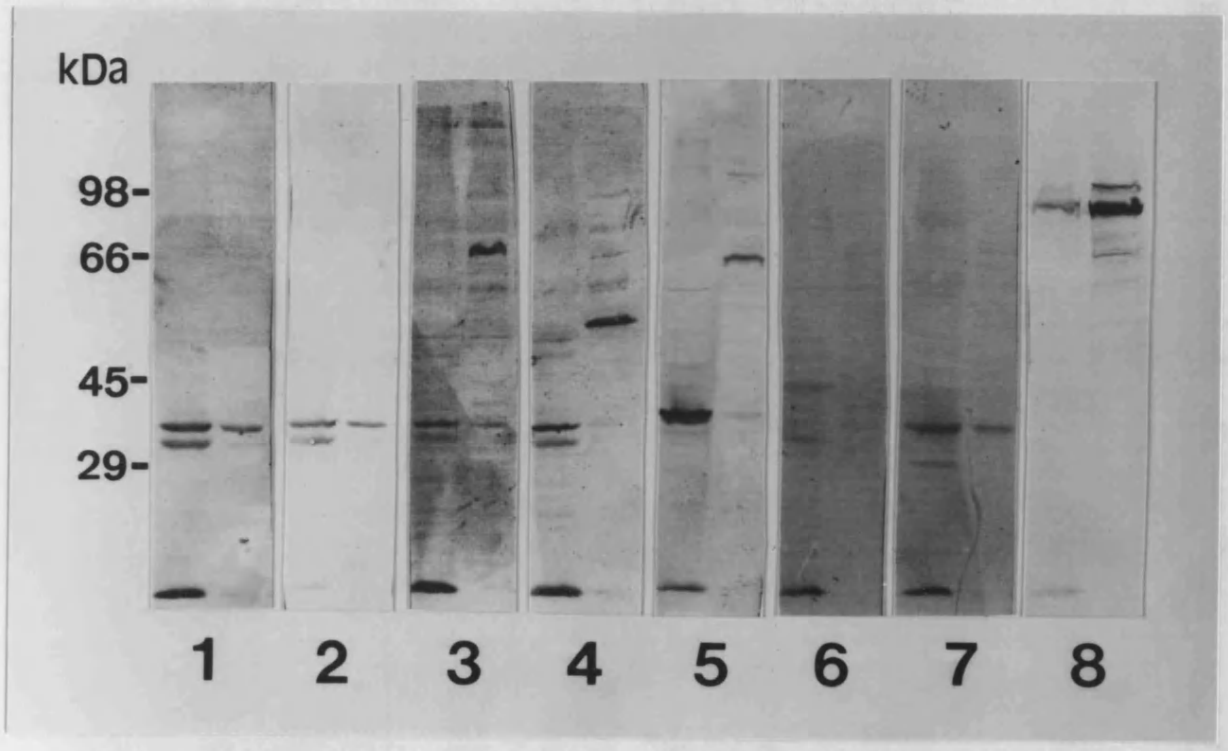


Fig 47 Western blot for annexins I, II, IV and V in total protein from transformed U937 cell lysates (left lane of panels) and platelet lysates (right lanes of panels). Proteins were separated on a one dimensional 10 % polyacrylamide SDS gel. Panels 1 to 4 respectively were probed for annexin I using four different anti-peptides to annexin I (amino acids 1-188; whole peptide; amino acids 24-37; amino acids 38-50 respectively). Panels 5,6 and 7 were probed for annexins II, IV and V respectively, whilst panel 8 has been probed using an antivinculin antibody as a positive control for platelets. This experiment is representative of three similar experiments.

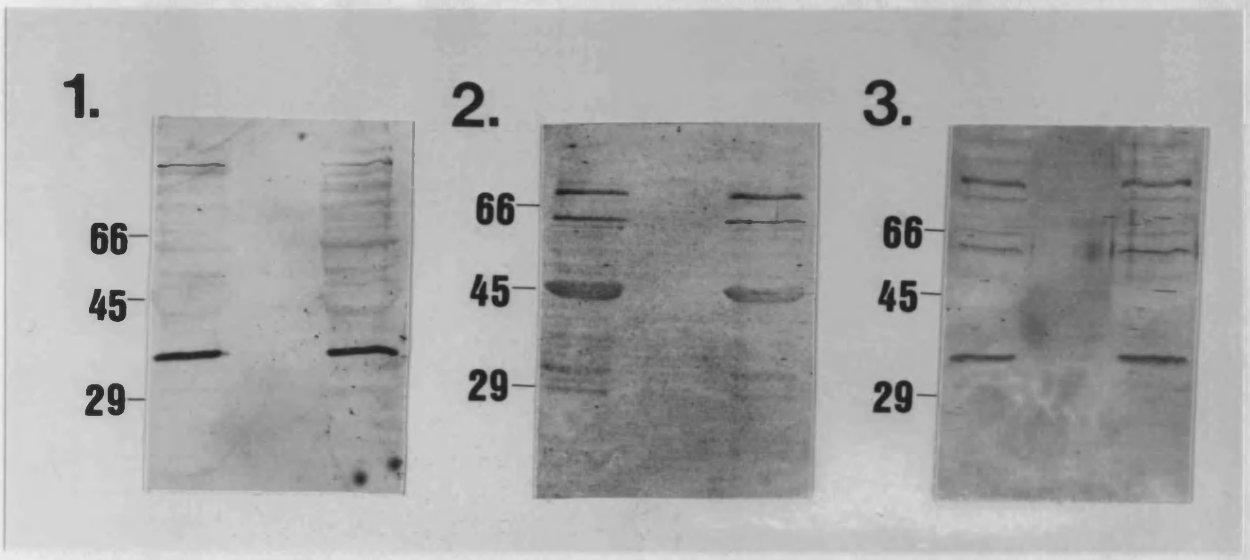


Fig 48 Western blots for annexins I, II and V of proteins associated with the external surface of the plasma membrane and released with EGTA. Panels 1,2 and 3 are Western blots for annexins I, II and V respectively. The left lane of each panel is annexin in total platelet lysates, the middle lane of each panel is annexin released from the surface of the plasma membrane by 5 mM EGTA/ 5 mM EDTA and the right lane is the annexin contained in the platelet pellet after removing any EGTA releasable external surface bound annexin. Anti-annexin antibodies were used at the concentrations described in section 2.2.13.1. Goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate was used as the 2° antibody. The blots are representative of three separate experiments.

Activation of platelets with the receptor operated agonists PAF (300 nM) and thrombin (0.3 unit/ml), or the divalent cation ionophore, ionomycin (5 μM) caused between 40% and 70% of dense granule release, whilst the PKC activator TPA caused only a small amount of release (Fig 50). The supernatant and the remaining platelet pellet were then probed by Western blotting for annexins I,II and V (Fig 49). Western blotting of the platelet supernatant and the remaining platelet pellet showed that neither the platelet activators nor the PKC activator caused release of annexins I,II or V into the supernatant suggesting that they are not located within dense granules in platelets. Platelet activation however, caused some degradation of annexin I to a lower molecular weight of 32 KDa which was most noticeable in platelets stimulated with ionomycin (Fig 49).

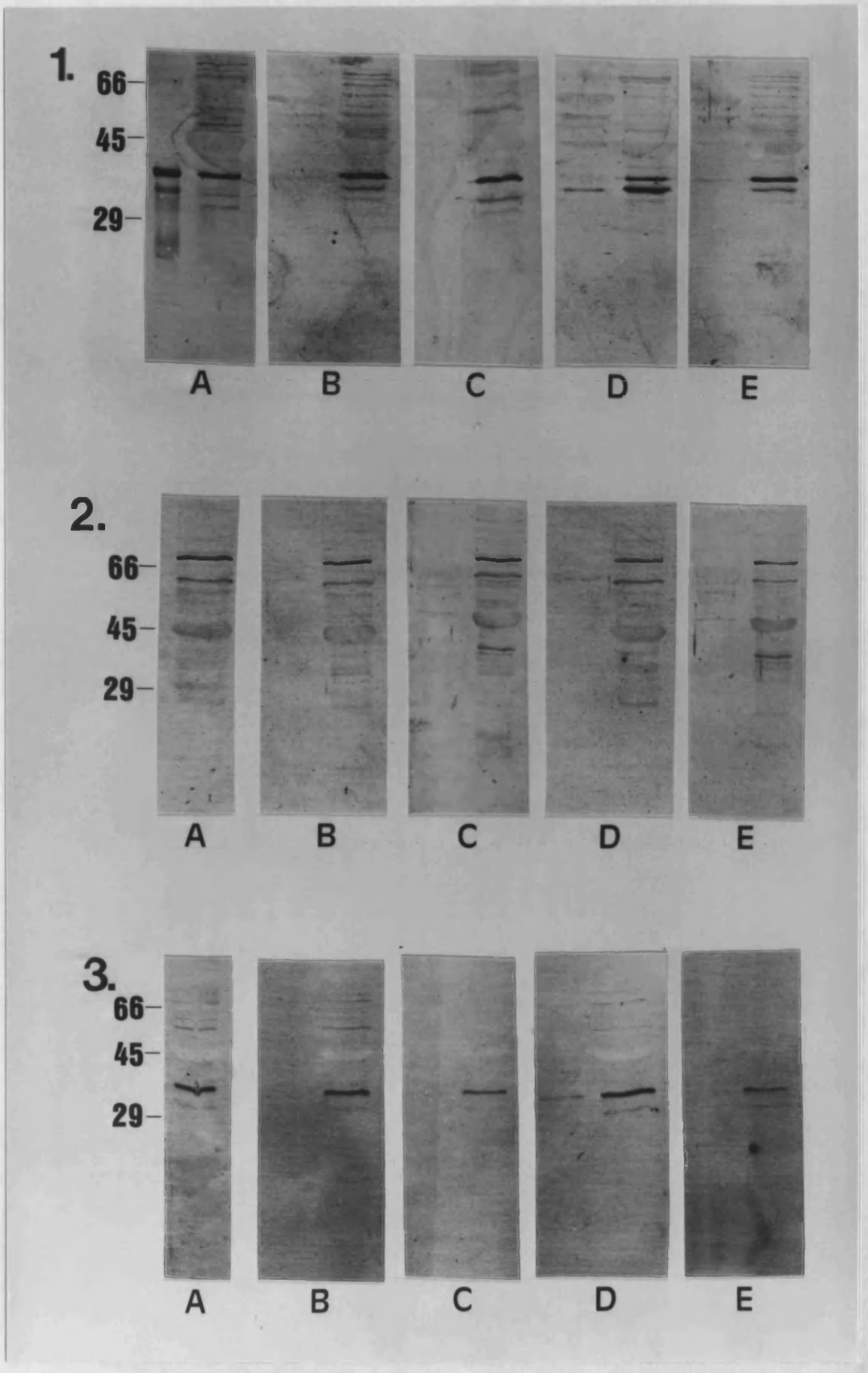
The whole annexin I peptide was used as a standard and is shown in Fig 49 (panel 1, track A) as a protein of 36 kDa. A final concentration of around 5 ng of the annexin 1 protein was added to the lane which gives an indication of the sensitivity of this Western blotting method.

3.5.3 The use of ELISA for determining the location of annexin 1

Using the ELISA method the concentration of annexin I contained in total platelet lysates, was found to be around 10 ng/10^9 platelets. No significant amount of annexin I (<0.1 ng/10^8 platelets) was released from the surface of platelets upon washing with the Ca^{++} chelators EGTA/EDTA.

The ELISA detection system was also used to investigate for the release of annexin I from the dense granules upon platelet stimulation with various platelet activators. Using ELISA the amount of annexin I released from platelets stimulated with PAF (300 nM), thrombin (0.3 units/ml), ionomycin (5 μM) or TPA (100 nM) was found to be <0.2 ng/10^9 platelets despite the fact that these treatments caused varying amounts of dense granule release (Fig 50).

Fig 49 Western blots of the granule releasate from activated platelets for annexins I, II and V. Panels 1,2 and 3 are Western blots probed for annexins I (amino acids 1-188), II and V respectively. Track A is annexin in total protein from unstimulated platelets (in panel 1 the annexin I peptide (5 ng) was run on the left side of track A as a control). Tracks B to E are granule releasate on the left with the remaining platelet pellet on the right for platelets stimulated with 300 nM PAF (track B), 0.3 units/ml thrombin (track C), 5 μM ionomycin (track D) and 100 nM TPA (track E). Anti-annexin antibodies are used at the concentrations described in section 2.2.13.1. The Western blots are representative of three separate experiments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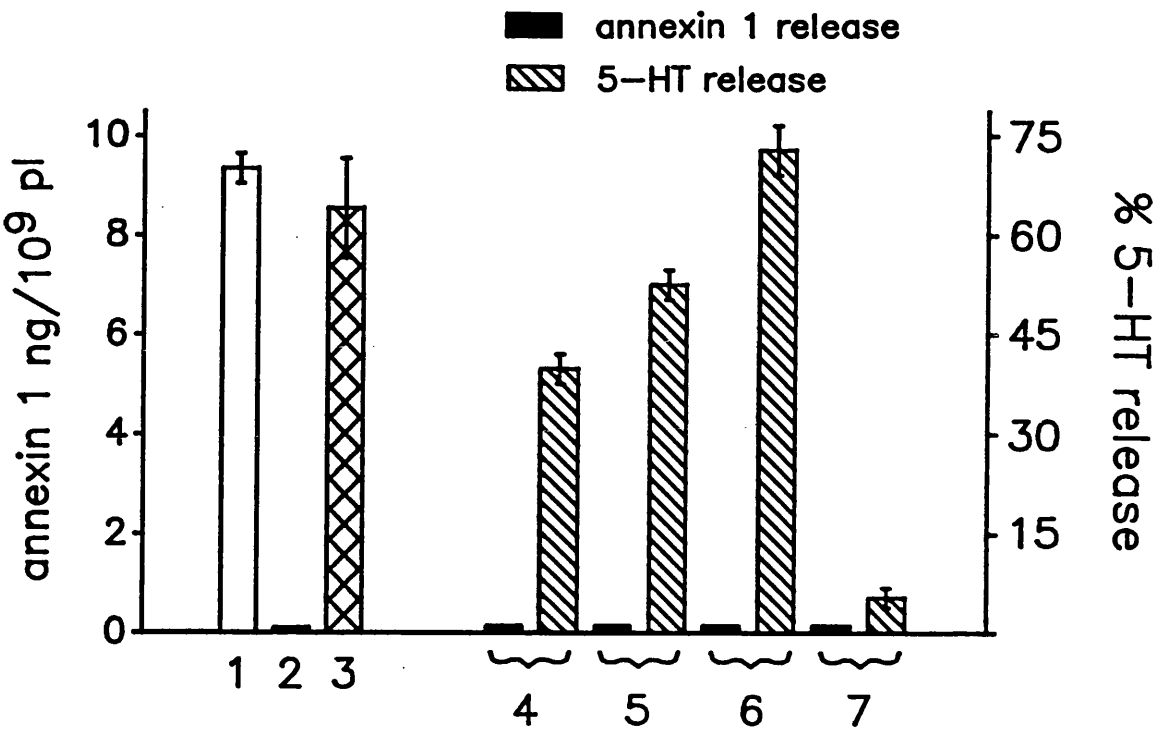

Fig 50 Comparison of ^{14}C-5HT release and of annexin I release in platelets treated with either PAF, thrombin, ionomycin or TPA. In column 1 is the total annexin 1 detected by ELISA in the total lysate of 10^9 platelets. Column 2 represents the annexin 1 detected in the supernatant of 10^9 platelets which had been treated with 5 mM EGTA/5 mM EDTA to remove external surface bound EGTA/EDTA releasable proteins, track 3 is the annexin 1 in the remaining platelet pellet after the treatment described for column 2. The four groups of columns numbered 4-7 are annexin I release (left column) and dense granule release (shown as % 5-HT release) (right column) of platelets stimulated with 300 nM PAF (group 4), 0.3 units/ml thrombin (group 5), 5 μM ionomycin (group 6) and 100 nM TPA (group 7). The values are determined from three different experiments each performed in triplicate and are given as the mean ± S.E.M.

It is possible that glucocorticosteroids (which induce the synthesis of annexin 1 in some nucleated cell types) may have another role and induce release of annexins. However, the introduction of 1 μM of the glucocorticoid steroid dexamethasone for 60 minutes had no effect on release of annexins, either in non-stimulated platelets or in platelets stimulated with ionomycin (results not shown).

3.6 Characterisation of tyrosine phosphorylation of proteins in PAF-stimulated platelets and the investigation of the role of endogenously activated tyrosine kinase(s) in the signal transduction pathway using the tyrosine kinase inhibitor genistein.

The presence of protein tyrosine kinase(s) (PTKase) in terminally differentiated cells such as platelets has only been recognised relatively recently. Their role is usually associated with cell growth and proliferation, however in platelets due to the rapidity of tyrosine phosphorylation upon receptor-agonist coupling it is possible that they have a role in signal transduction unrelated to proliferation.

3.6.1 PAF-induced tyrosine phosphorylation in intact platelets

Western blotting using the monoclonal anti-phosphotyrosine antibody (PY20) (Glenney *et al.*1988) detected at least 4 tyrosine phosphorylated proteins between the molecular weights of 52 - 62 kDa in unstimulated platelets (Fig 51). Within 5 seconds of platelet stimulation with 300 nM PAF phosphorylation of two groups of proteins between the molecular weight ranges 35 - 45 kDa and 66 - 90 kDa was induced. Within 30 seconds after 300 nM PAF stimulation a third molecular weight range of tyrosine phosphorylated proteins between 90 - 150 kDa was detected. It appears therefore that there are three sets of phosphorylation, firstly, tyrosine phosphorylation in unstimulated platelets (mw 52 - 62 kDa), secondly, tyrosine phosphorylation 2 - 5 seconds after PAF stimulation (mw 35 - 45 kDa and 66 - 90 kDa) and thirdly tyrosine phosphorylation 15 - 30 seconds after PAF stimulation (mw 90 - 150 kDa) (Fig 51).

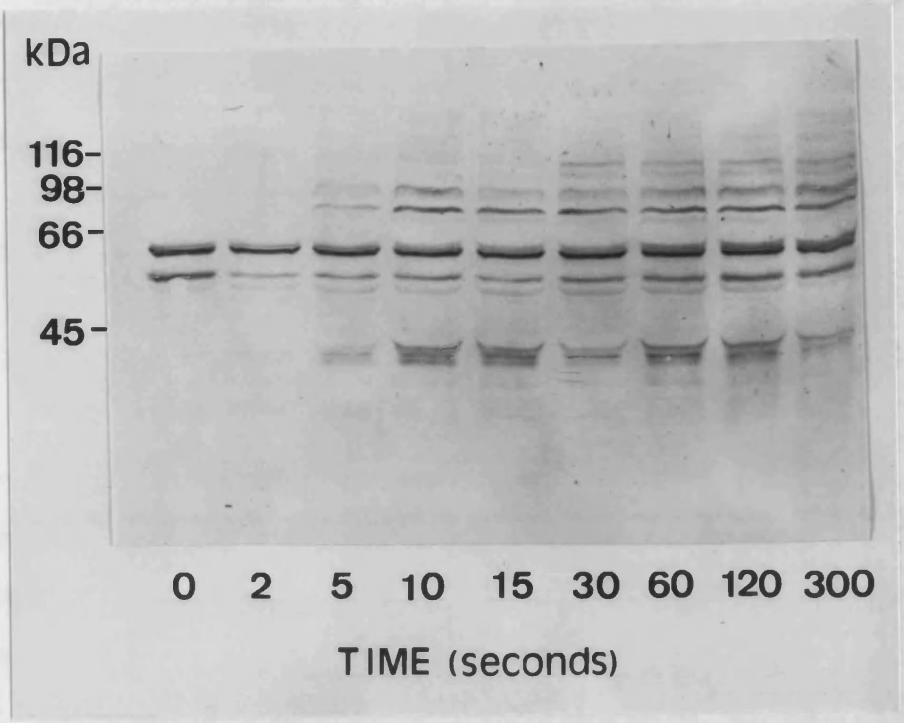

Fig 51 Time course of PAF-induced tyrosine phosphorylation in intact platelets. Western blot of tyrosine phosphorylated proteins from platelets stimulated with 300 nM PAF over a 5 minute time course. Platelet proteins were separated on a one dimensional 9.5% polyacrylamide SDS gel. A 1:500 dilution of anti-phosphotyrosine (PY20) monoclonal antibody was used as the 1° antibody, a 1:500 dilution of goat anti-mouse IgG horseradish peroxidase conjugate was used as the 2° antibody and the blots were enhanced using a 1:10,000 dilution of peroxidase anti-peroxidase antibody (mouse). The blot shown is representative of 4 similar experiments.

At least 17 tyrosine phosphorylated proteins were detected by Western blotting using the monoclonal anti-phosphotyrosine antibody PY20, however, as this system used a one dimensional SDS gel, tyrosine phosphorylation of proteins of the same molecular weight were not differentiated from each other, and therefore the total number of tyrosine phosphorylated proteins could be higher.

Comparing the total relative optical density of each of the four groups of tyrosine phosphorylated proteins over a two minute time course (Fig 52) the phosphorylation and dephosphorylation patterns of each group can be examined. 300 nM PAF-stimulation caused a noticeable dephosphorylation of the group of proteins (52-62 kDa) originally detected in non-stimulated platelets. However, over the two minute time course the proteins returned to their original level of phosphorylation. The two groups of proteins 35-45 kDa and 66-90 kDa were rapidly phosphorylated upon PAF-stimulation followed in both groups by a degree of dephosphorylation. In the group of proteins 90-150 kDa examination of the total relative optical density demonstrated a lag phase before phosphorylation could be detected, after which there was rapid phosphorylation followed by a plateau phase.

3.6.2 Validation of the technique for detection of phosphotyrosine

It was important to confirm that the proteins being detected on the Western blots were tyrosine phosphorylated and not serine or threonine phosphorylated. As demonstrated in Fig 53 excess phosphotyrosine (1mM) added to the primary antibody incubation (ie anti-phosphotyrosine antibody) completely blocked the anti-phosphotyrosine antibody (PY20) from binding to tyrosine phosphorylated proteins induced by PAF over a 5 minute time course. Conversely addition of 1 mM phosphoserine and 1 mM phosphothreonine had no effect on the phosphorylation pattern induced by PAF over a 5 minute time course when compared to Fig 51. These results strongly suggest that all the protein bands detected using PY20 are tyrosine phosphorylated and that the antibody does not cross react with either phosphoserine or phosphothreonine.

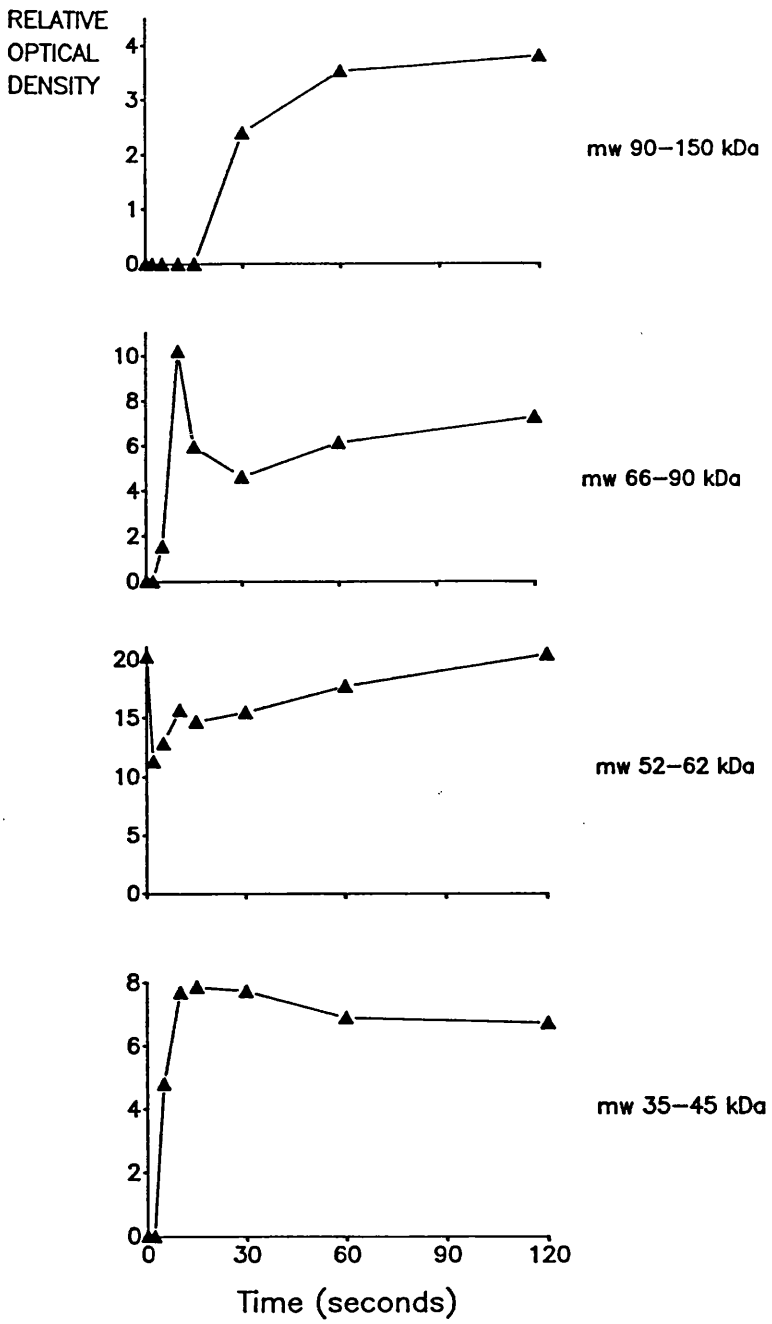

Fig 52 Optical densitometry of PAF-induced tyrosine phosphorylation in intact platelets over a five minute time course. The Western blots of the time course of tyrosine phosphorylation in 300 nM PAF stimulated platelets were scanned by optical densitometry. Each of the four figures represents the sum of the relative optical densities of all the proteins contained in one of the four different groups of proteins detected either in unstimulated platelets (52-62 kDa) or detected upon platelet activation with 300 nM PAF (35-45 kDa; 66-90 kDa; 90-150 kDa) over a two minute time couse. The data are taken from one experiment which is representative of four similar experiments.

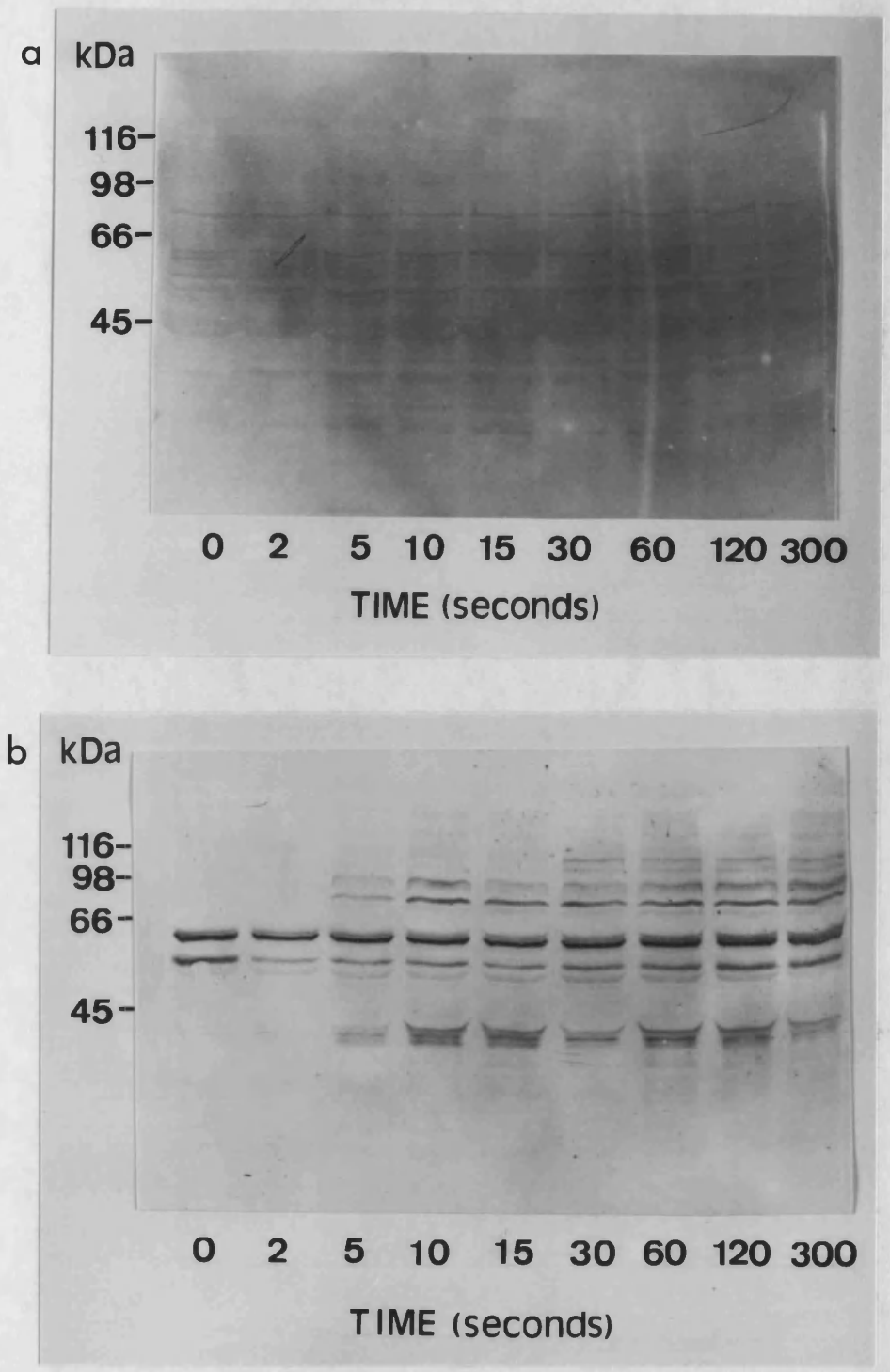

Fig 53 Effect of free phosphotyrosine (a) or free phosphoserine/phosphothreonine (b) on binding of antiphosphotyrosine antibody to nitrocellulose bound tyrosine phosphorylated proteins. Both the Western blots (a) and (b) were performed using the same protein samples representing a 5 minute time course after platelet stimulation with 300 nM PAF. Conditions were as described in Fig 51, except incubation with the 1° antibody was carried out in the presence of either 1 mM phosphotyrosine (a) or 1 mM phosphoserine and 1 mM phosphothreonine (b). Each blot shown is representative of 2 similar experiments.

3.6.3 Dose response relationship of PAF-induced tyrosine kinase phosphorylation

Increasing concentrations of PAF (0.3 - 300 nM) caused a dose dependent increase in phosphorylation of PTKase substrates (Fig 54) when measured at 30 seconds after PAF-induced platelet activation.

3.6.4 Effect of genistein on tyrosine kinase activity

The effect of genistein on PTKase activity was determined by investigating the effect of genistein on phosphorylation of the substrates of PTKase (Fig 55). Platelets were pretreated with a range of concentrations of genistein for 20 minutes before activation with 300 nM PAF. Genistein (300 μM) alone had no effect on tyrosine phosphorylation of the substrates of PTKase normally found in unstimulated platelets. Genistein (30 - 1000 μM) did however dose dependently inhibit tyrosine phosphorylation of several of the protein substrates of PTKase(s) whose phosphorylation was induced by 300 nM PAF. The most noticeable inhibition by genistein were the groups of proteins of the molecular weight ranges 35 - 45 kDa and 68 - 90 kDa. Genistein had less effect on phosphorylation of the proteins of the 90 - 150 kDa molecular weight range. It was possible to examine the extent to which phosphorylation of each of the 17 tyrosine phosphorylated proteins detected was inhibited by genistein with the use of relative optical densitometry (Fig 56). Scanning the Western blot demonstrated that at a concentration of 300 μM genistein phosphorylation of the proteins in the groups 35-45kDa and 66-90 kDa had been inhibited by 90%, whilst phosphorylation of proteins in the group 90-150 kDa had been inhibited to a lesser extent of 50%. The tyrosine phosphorylated proteins which were first observed in unstimulated platelets were not significantly inhibited by the pre-incubation with 300 μM genistein.

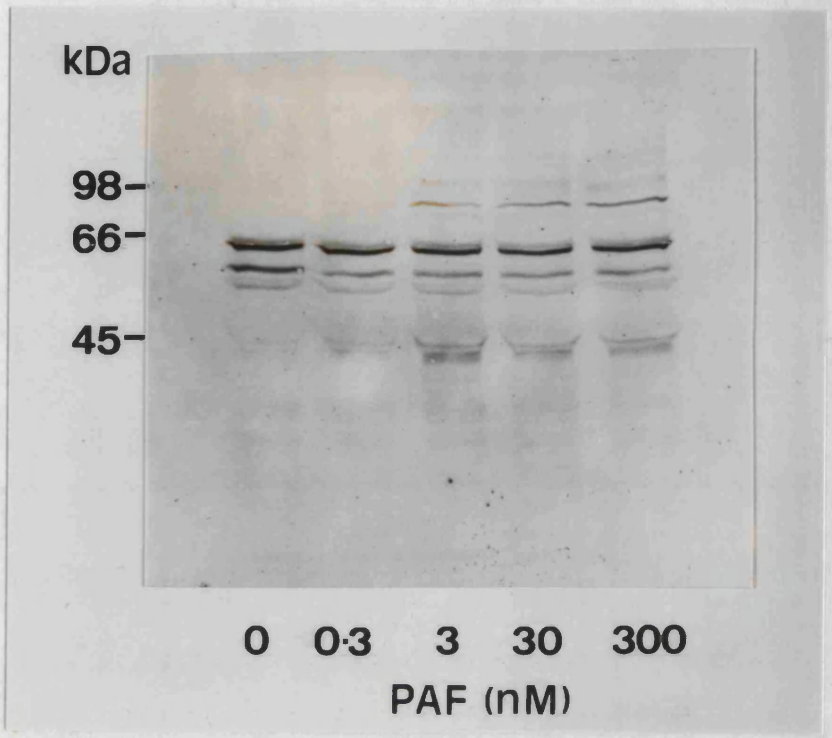

Fig 54 Effect of increasing concentrations of PAF on tyrosine phosphorylation. Platelets were stimulated with 0, 0.3, 3, 30 and 300 nM PAF and activation terminated at 30 seconds post-PAF. Platelet proteins were separated on a one dimensional 9.5% polyacrylamide SDS gel. A 1:500 dilution of anti-phosphotyrosine (PY20) monoclonal antibody was used as the 1° antibody, a 1:500 dilution of goat anti-mouse IgG horseradish peroxidase conjugate was used as the 2° antibody. The blot shown is representative of 3 similar experiments.

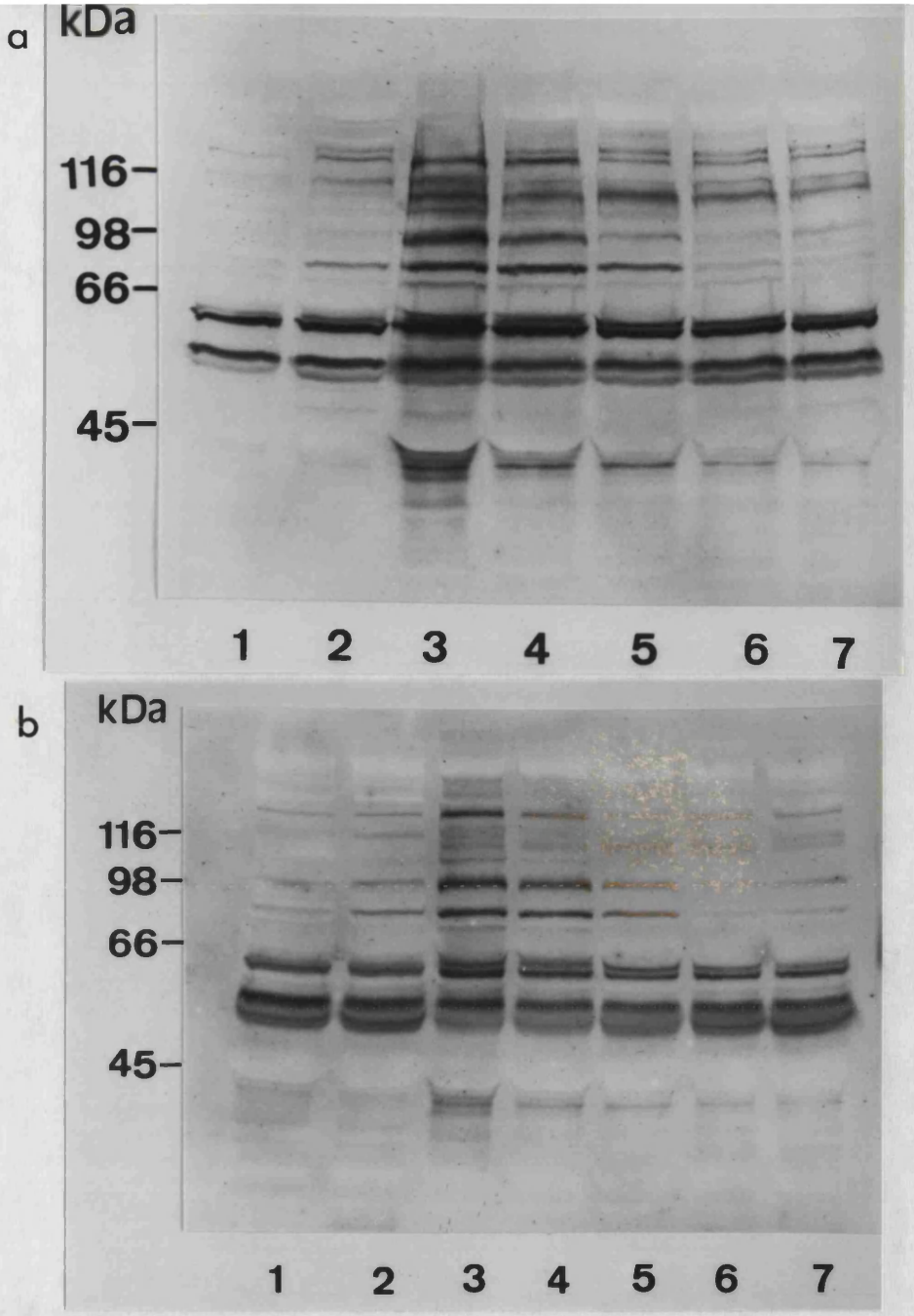

Fig 55 The effect of genistein on PAF-induced tyrosine phosphorylation. Platelets were pretreated with either genistein (30, 100, 300, 1000 μM) or genistein vehicle for 20 minutes prior to platelet activation with 300 nM PAF. Platelet activation was terminated at 30 seconds post-PAF. In both panels platelet proteins were separated on a one dimensional 9.5% polyacrylamide SDS gel. In the upper panel a 1:500 dilution of anti-phosphotyrosine (PY20) monoclonal antibody was used as the 1° antibody, a 1:500 dilution of goat anti-mouse IgG horseradish peroxidase conjugate was used as the 2° antibody and the blots were enhanced using a 1:10,000 dilution of peroxidase anti-peroxidase antibody. In panel (b) a 1:500 dilution of antiphosphotyrosine polyclonal antibody was used as the 1° antibody whilst a 1:500 dilution of goat anti-rabbit IgG horse radish peroxidase conjugate was used as the 2° antibody, and the blot enhanced using a 1:10,000 dilution of peroxidase anti-peroxidase antibody. Each Western blot shown is representative of 4 separate experiments.

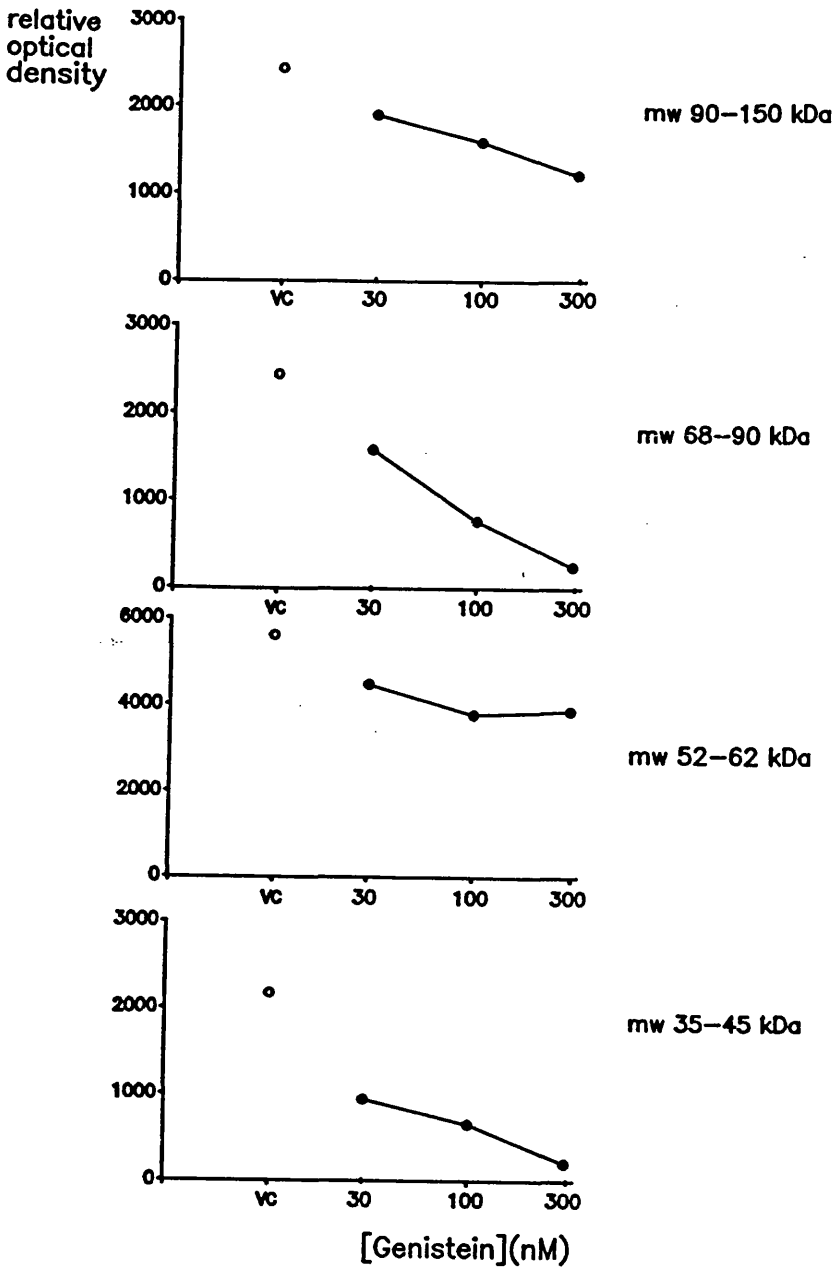

Fig 56 Densitometry of the tyrosine phosphorylated proteins in platelets pretreated with genistein. Western blots (probed using monoclonal anti-phosphotyrosine antibody) demonstrating the effect of a dose range of genistein on tyrosine phosphorylation (as described in Fig 48) were scanned by optical densitometry. Each of the four figures represents the sum of the relative optical densities of all the proteins contained in one of the four different groups of proteins detected either in unstimulated platelets (52-62 kDa) or detected upon platelet activation with 300 nM PAF (35-45 kDa; 66-90 kDa; 90-150 kDa).

3.6.5 The use of a polyclonal anti-phosphotyrosine antibody

It was also important to consider the possibility that a polyclonal anti-phosphotyrosine antibody would have a more inclusive recognition of all phosphotyrosine containing proteins than a monoclonal antibody. The polyclonal preparation by definition, a family of antibodies may recognise more phosphotyrosine containing proteins than a monoclonal, particularly when the amino acid sequence in the vicinity of the phosphotyrosine influences the immunoreactivity of the epitope itself. However, using a polyclonal anti-phosphotyrosine antibody, the same number of tyrosine phosphorylated proteins were detected in total platelet proteins as when using the monoclonal anti-phosphotyrosine antibody and genistein inhibited phosphorylation of the same molecular weight protein bands (Fig 55). The only difference in the Western blots probed using either a monoclonal or a polyclonal anti-phosphotyrosine antibody was that the relative density of some of the proteins varied with the antibody used. The pattern of genistein inhibition of tyrosine phosphorylated proteins was similar in Western blots probed with the monoclonal antibody or the polyclonal antibody as is shown in Fig 55.

3.6.6 Effect of genistein on PAF induced signal molecule elevation and platelet functional responses

Using the range of concentrations of genistein which inhibited PTKase(s), the effect of preincubation with genistein on signal molecule production and platelet functional responses induced by PAF were investigated.

3.6.6.1 Effect of Genistein on Ins(1,4,5)P_3 production

A 20 minute preincubation with genistein (300 μM) had no effect on the basal level of Ins(1,4,5)P_3 which was 8.5 pmol/10^9 platelets (Fig 57). The effect of inhibition of PTKase(s) was investigated on Ins(1,4,5)P_3 levels in platelets 5 seconds after stimulation with 300 nM PAF as at this time after PAF stimulation Ins(1,4,5)P_3 levels were known to be significantly elevated above basal (see Fig 26). Increasing concentrations of genistein caused a dose dependent inhibition of Ins(1,4,5)P_3 elevation, with 300 μM genistein completely inhibiting any elevation of Ins(1,4,5)P_3 above basal. The IC_{50} for inhibition of Ins(1,4,5)P_3 elevation with genistein was 80 μM.

3.6.6.2 Effect of Genistein on $[Ca^{++}]_i$ elevation

As Ins(1,4,5)P_3 is believed to be the signal molecule which is responsible for release of Ca^{++} from intracellular stores, the effect of genistein on Ca^{++} mobilization from intracellular stores was investigated.

For these experiments platelets were loaded with the fluorescent dye fluo-3-AM instead of fura-2-AM, as initial experiments indicated that genistein was interfering with the fluorescence signal at the wavelengths required to measure emission of fura-2 fluorescence. In the presence of 1 mM $[Ca^{++}]$ the fluorescence signal from fura-2 free acid but not fluo-3 free acid was reduced by the addition of genistein.

To investigate the effect of genistein on Ca^{++} mobilization from intracellular stores platelets were suspended in HBT where the $[Ca^{++}]_e$ had been replaced by 2 mM of the Ca^{++} chelator EGTA (Fig 58). Stimulation of platelets with 3 nM PAF caused a transient increase of $[Ca^{++}]_i$ above basal due to release of Ca^{++} from the intracellular stores. Pretreatment with genistein had no effect on basal levels of $[Ca^{++}]_i$, but as with Ins(1,4,5)P_3 elevation caused a dose dependent inhibition of $[Ca^{++}]_i$ mobilization induced by PAF with an IC_{50} of 120 μM, similar to that found for the inhibition of Ins(1,4,5)P_3 by genistein.

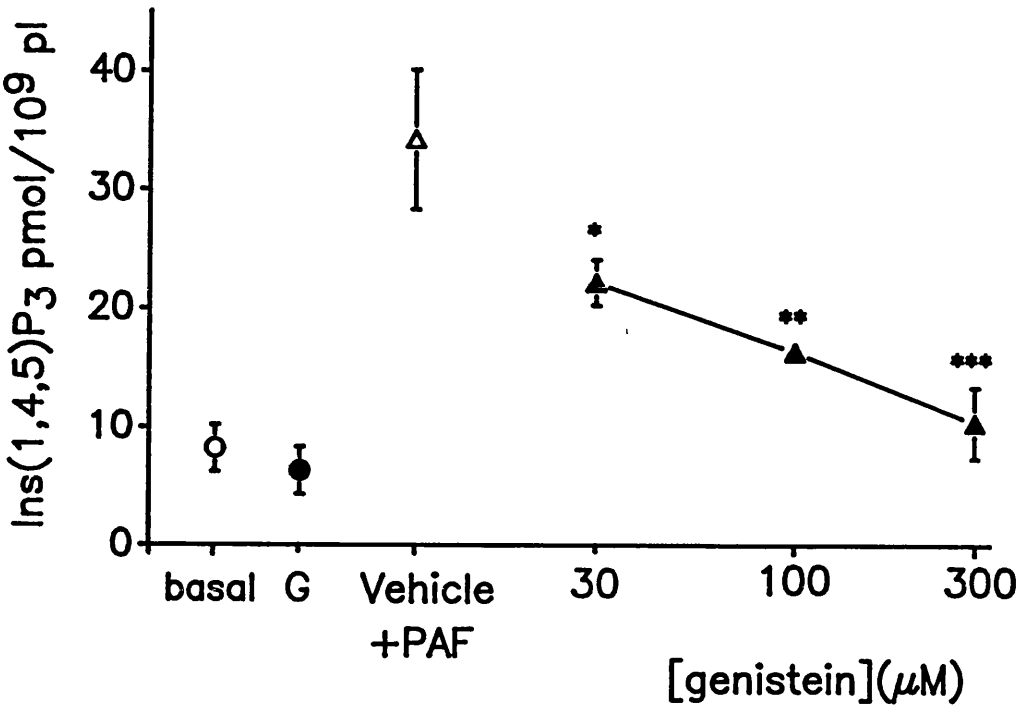

Fig 57 Effect of genistein on the basal level of Ins (1,4,5)P_3 or PAF-induced Ins(1,4,5)P_3 elevation. Platelets were pre-treated with either genistein vehicle or genistein (30, 100, 300 μM) for 20 minutes prior to activation with 300 nM PAF. Platelet activation was terminated at 5 seconds post-PAF. The values are the mean ± S.E.M. of 4 separate experiments each performed in duplicate.

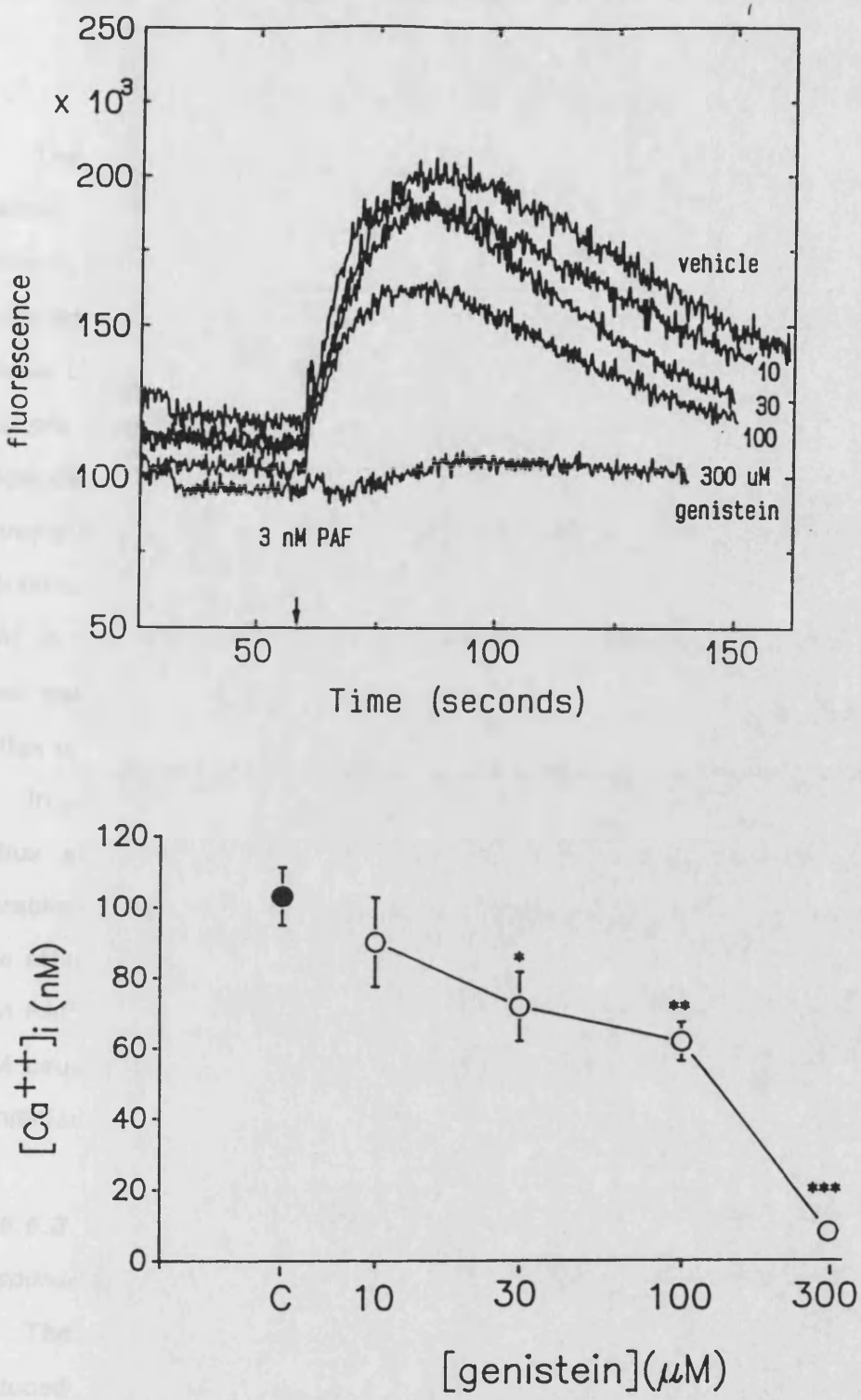

Fig 58 Effect of genistein on 3 nM PAF-induced $[Ca^{++}]_i$ elevation in platelets in the presence of 1 mM EGTA. Platelets were pretreated for 20 mins with genistein vehicle or genistein before activation with 3 nM PAF. Left: fluorescent output from fluo-3 loaded platelets. Right: fluorescence has been converted to $[Ca^{++}]_i$. $[Ca^{++}]_i$ is the peak elevation of cytosolic calcium in fluo-3 loaded platelets after the basal pre-PAF value has been subtracted. The traces are taken from a single experiment and the data for the right figure are the mean $\pm$ S.E. of three separate experiments each performed in triplicate.

The effect of inhibiting PTKase(s) on both components of $[Ca^{++}]_i$ elevation, namely Ca^{++} influx and Ca^{++} mobilization from intracellular stores in platelets stimulated with 3 nM PAF was investigated in the presence of 1 mM $[Ca^{++}]_e$. Stimulation of platelets with 3 nM PAF induced a transient increase in $[Ca^{++}]_i$ above basal levels. Genistein (300μM) had no effect on basal levels of $[Ca^{++}]_i$, but pre-incubation with a dose range of genistein from 10-300 μM caused a dose dependent inhibition of $[Ca^{++}]_i$ elevation induced by 3 nM PAF, with almost complete inhibition of $[Ca^{++}]_i$ elevation in platelets pretreated with 300 μM genistein (Fig 59). The IC_{50} for the inhibition of $[Ca^{++}]_i$ elevation induced by 3 nM PAF in the presence of 1 mM $[Ca^{++}]_e$ was 100 μM genistein. It therefore appears that genistein is inhibiting both Ca^{++} mobilization from intracellular stores, and influx of Ca^{++} across the plasma membrane.

In platelets stimulated with 0.3 nM PAF $[Ca^{++}]_i$ elevation is due solely to Ca^{++} influx and not Ca^{++} mobilization (see Fig 17). Therefore in order to further establish whether the influx component of $[Ca^{++}]_i$ was also inhibited by genistein, the effect of genistein on $[Ca^{++}]_i$ was investigated in platelets stimulated with 0.3 nM PAF. Pre-activation of platelets with a dose range of genistein from 30 - 300 μM caused a dose dependent inhibition of $[Ca^{++}]_i$ elevation (Fig 60). The IC_{50} for inhibition of influx of Ca^{++} induced by 0.3 nM PAF by genistein was 60 μM.

3.6.6.3 Effect of inhibiting tyrosine kinase(s) on PAF-induced platelet functional responses

The effect of inhibiting PTKase(s) with genistein was investigated on PAF-induced TxB_2 generation, 5-HT release (Fig 61) and platelet aggregation (Fig 62). Pre-incubation of platelets with genistein (300 μM) did not induce TxB_2 generation, nor did it cause any release of dense granule constituents (Fig 61). The presence of genistein vehicle also had no affect on either 5-HT release or TxB_2 generation induced by suboptimal concentrations of PAF. Platelets were pre-incubated with a dose range of genistein (10 - 300 μM) for 20 minutes before stimulation with either 3 nM PAF (dense granule release) or 30 nM PAF (TxB_2 generation).

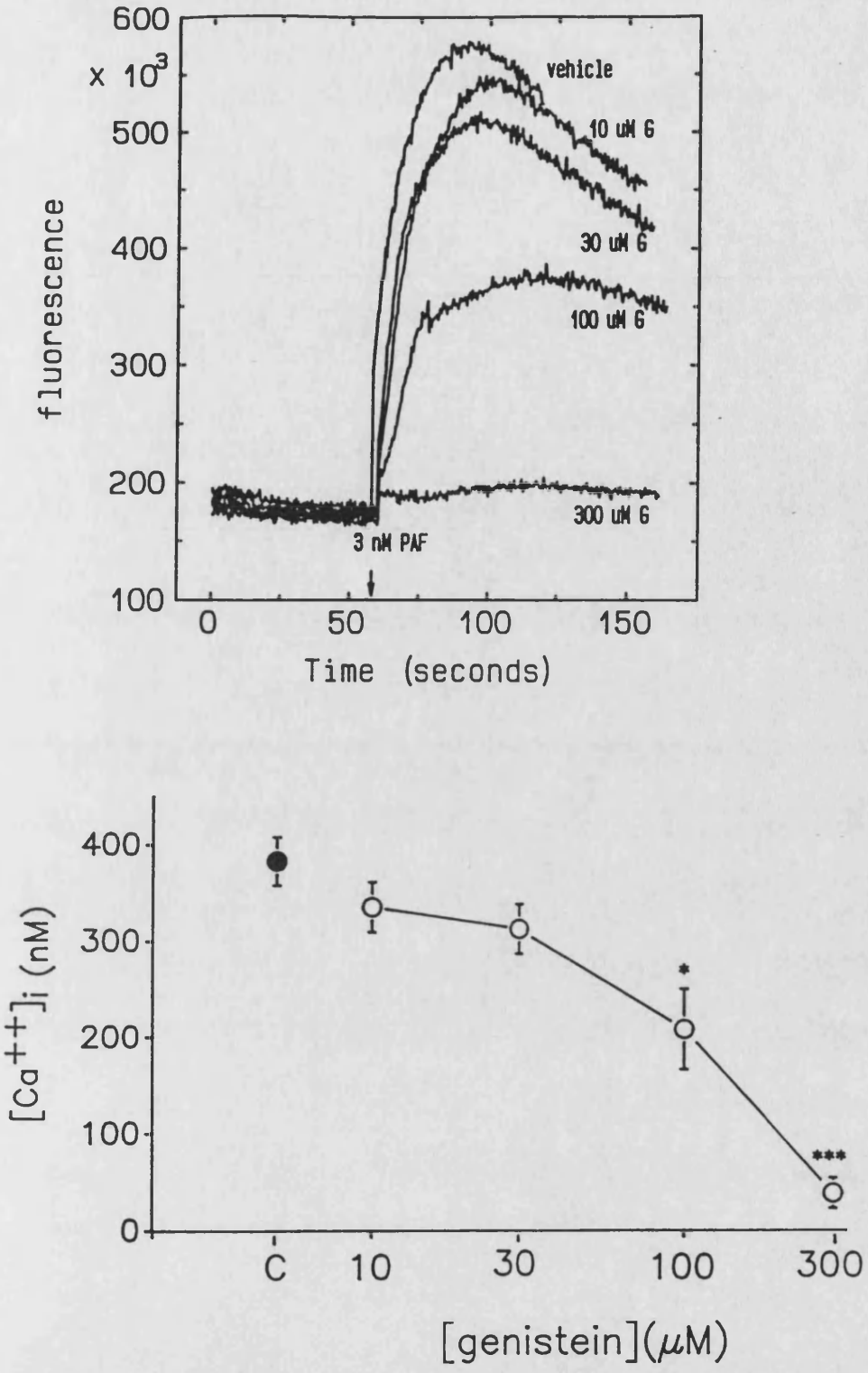

Fig 59 Effect of genistein on 3 nM PAF-induced $[Ca^{++}]_i$ elevation in platelets in the presence of 1 mM $[Ca^{++}]_e$. On the left is the fluorescent output from fluo-3 loaded platelets, on the right the fluorescence has been converted to $[Ca^{++}]_i$. $[Ca^{++}]_i$ is the peak elevation of cytosolic calcium in fluo-3 loaded platelets after the basal pre-PAF value has been subtracted. The traces are taken from a single experiment and the data from the figure on the right are the mean $\pm$ S.E. of three separate experiments each performed in triplicate.

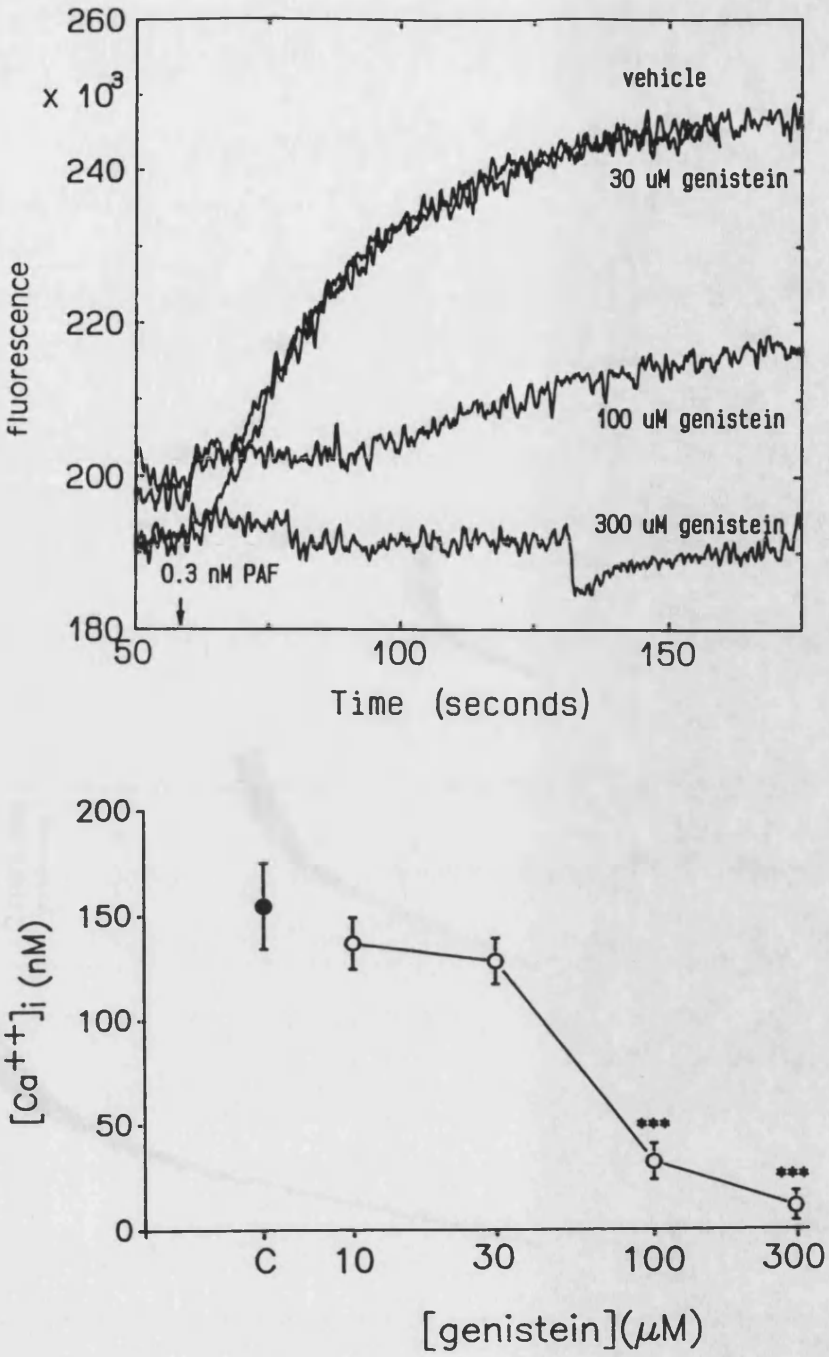

Fig 60 Effect of genistein on 0.3 nM PAF-induced $[Ca^{++}]_i$ elevation. Platelets were pretreated with either genistein vehicle or genistein (10, 30, 100, 300 μM) for 20 minutes before platelet activation with 0.3 nM PAF. The traces in the upper panel represent the fluorescent output of fluo-3 loaded platelets and are from an individual experiment. In the lower panel fluorescence has been converted to $[Ca^{++}]_i$ which is the peak $[Ca^{++}]_i$ elevation after the pre-PAF value has been removed. The data are taken from two separate experiments each performed in triplicate.

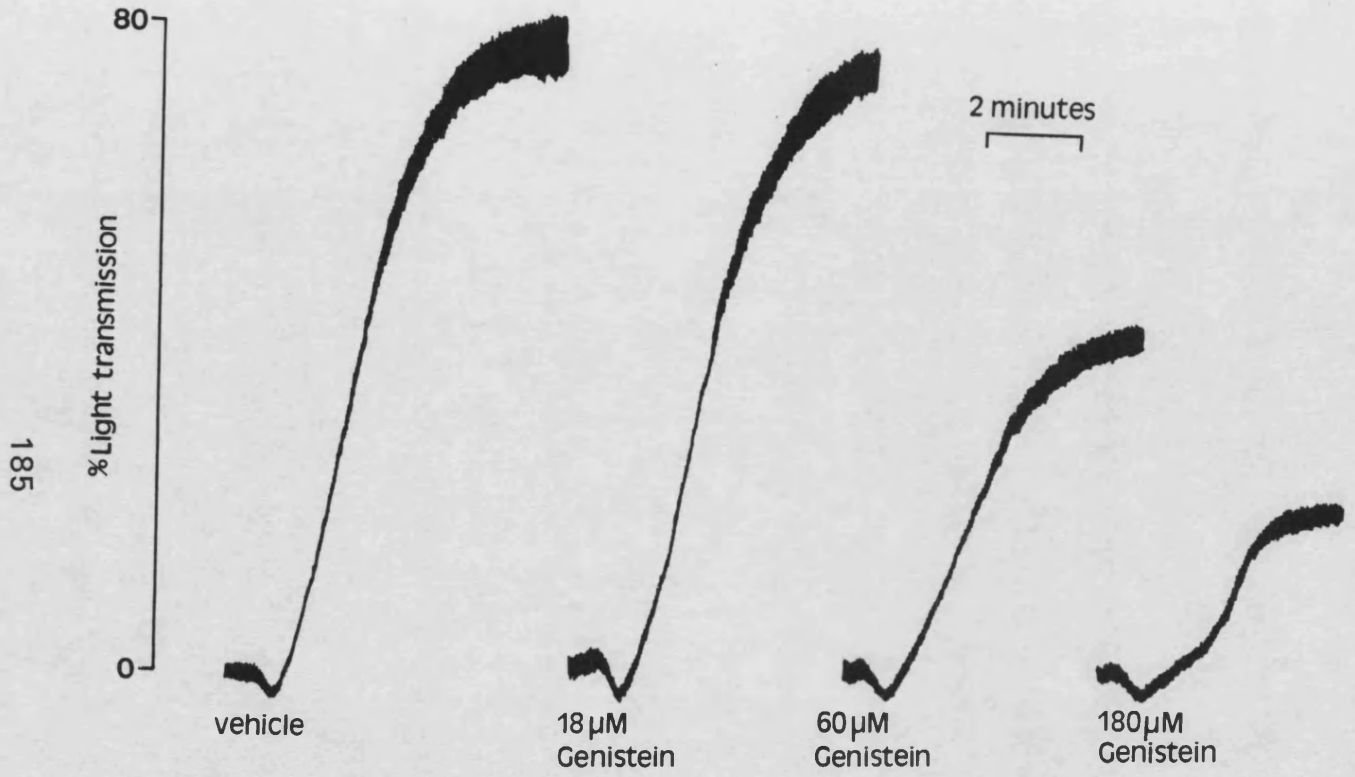

Fig 62 The effect of genistein on PAF-induced platelet aggregation. Platelets were pre-treated with genistein for 20 minutes before platelet aggregation was induced with 1 nM PAF. The traces are from an individual experiment but are representative of four similar experiments each performed in triplicate.

Inhibition of PTKase(s) with genistein caused a dose dependent inhibition of 5-HT release with an IC_{50} of 60 μM close to the value found for both inhibition of $Ins(1,4,5)P_3$ and Ca^{++} elevation (see Figs 57 & 58). Genistein also caused a dose dependent inhibition of TxB_2 generation but with an IC_{50} of around 10 μM (Fig 60).

In addition, genistein vehicle had no affect on 1 nM PAF induced platelet aggregation whereas a 20 minute pretreatment with a range of concentrations of genistein inhibited platelet aggregation (Fig 62). The IC_{50} of 120 μM genistein for the inhibition of 1 nM PAF-induced aggregation was similar to that found for the inhibition of $Ins(1,4,5)P_3$ elevation and $[Ca^{++}]_i$ elevation.

4. DISCUSSION

4.1 Prostacyclin washed platelets

Platelets have receptors for PGI_2 which when stimulated by receptor-PGI_2 coupling cause activation of adenylate cyclase and increase cyclic adenosine monophosphate (cAMP) which activates PKA (Whittle *et al.*1985). Activation of this signal transduction pathway and the accumulation of cAMP causes an inhibition of platelet functional responses including elevation of $[Ca^{++}]_i$, aggregation, granule release and prostaglandin generation. Elevation of cAMP is transient due to its removal from the system by cAMP phosphodiesterase and as cAMP is removed the physiological responses of platelets to agonists return (Blackwell *et al.*1982b).

In this study platelets have been washed in the presence of PGI_2 according to the method of Blackwell *et al* (Blackwell *et al.*1982b). Their study demonstrated that addition of PGI_2 during the washing procedure inhibited platelet activation during platelet preparation (measured as TxB_2 generation and liberation of labelled oleate from intracellular lipid stores) but that once platelets were resuspended in PGI_2 free HBT, cAMP levels declined rapidly reaching basal between 90 and 120 minutes later with full functional activation restored. This method of platelet isolation also improved platelet viability and maintained platelet sensitivity to aggregating agents over a longer period of time after washing compared to platelets washed in the absence of PGI_2.

Washed platelets were used in this study, as opposed to PRP, because the responses of platelets in plasma are reduced due to binding of the agonist to plasma proteins. The use of washed platelets allows the manipulation of extracellular ion concentrations and in these investigations allowed the concentration of extracellular Ca^{++} to be modified. Moreover, studies using the fluorescent dye fura-2 need to be done in the absence of plasma proteins which interfere with the fluorescence signal of fura-2. Furthermore, most of the work carried out in platelet research now uses washed platelets, therefore in order for the work in this study to be comparable to other published investigations, it was important to use a similar method of platelet

preparation. Rabbit platelets were used for the majority of the investigations reported here. PAF is a potent agonist for rabbit platelets (Hwang *et al.*1983; Morrison and Shukla, 1989) and many investigations concerning the study of signal transduction have been performed using rabbit platelets (Morrison and Shukla, 1989; Shukla *et al.*1987). The use of rabbit platelets rather than human alleviated the problems caused in the recruitment of human donors and the handling and disposal of human blood. Moreover using rabbit blood removed the variability which could be encountered when working with human blood, such as whether the donor smoked, whether they were on any drugs, the sex of the donor and any differences in diet and exercise between individual donors all of which could potentially affect platelet responses.

4.2 The role of protein phosphorylation in platelet activation

4.2.1 Comparison of the role of protein kinase C in platelet functional responses induced by three different mechanisms PAF, ionomycin and arachidonic acid

Platelets possess membrane receptors for the agonist PAF and, as demonstrated in this study, PAF-receptor coupling elicits a wide range of functional responses, which are likely to be triggered at different stages of the intracellular signal transduction pathway. In this study we have demonstrated that PAF induces a dose dependent elevation in $[Ca^{++}]_i$ elevation, TxB_2 generation and dense granule release. Alternatively, in the presence of extracellular Ca^{++}, divalent cation ionophores also elicit a wide range of functional responses in platelets which are due to the increase in $[Ca^{++}]_i$. Activation of platelets by directly increasing $[Ca^{++}]_i$ with the use of ionophores has been shown to be independent of PLC activation (ie activation of PLC is dependent upon cyclo-oxygenase products) (Rittenhouse, 1984; Halenda *et al.*1989) therefore providing a mechanism of activating the signal transduction pathway downstream of PLC. In this study we demonstrate that ionomycin can induce a dose-dependent elevation in $[Ca^{++}]_i$, TxB_2 generation and dense granule release. Platelets do not possess membrane receptors for AA, but it is readily metabolised by the cyclo-oxygenase pathway, for which the most abundant

product is TxA_2 (Hamberg *et al.*1975). Platelets do however possess membrane receptors for TxA_2 which is a potent platelet agonist and platelets can also be activated by PGH_2, the precursor of TxA_2 (Morinelli *et al.*1987). AA therefore provides a mechanism for generation of Tx, which is downstream of both PLC and PLA_2. A schematic drawing of the different mechanisms of platelet activation by the three agents PAF, ionomycin and AA is given in figure 63.

It is well established that activation of PKC followed by platelet stimulation with a variety of agonists causes an inhibition of $[Ca^{++}]_i$ elevation (Poll and Westwick, 1986a; Zavoico *et al.*1985). In agreement, the findings of this study demonstrate that pretreatment of platelets with the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (TPA) which directly activates PKC by substituting for DAG and increasing the affinity of the enzyme for Ca^{++} and for phospholipid (Castagna *et al.*1982), causes an inhibition of the subsequent $[Ca^{++}]_i$ elevation in platelets stimulated by PAF. The non-esterified precursor molecule of TPA, namely 4ß-phorbol (at a concentration of 1000 fold greater than that found to be active for TPA) was however ineffective at inhibiting PAF-induced $[Ca^{++}]_i$ elevation, inferring that PKC is involved in the modulation of $[Ca^{++}]_i$ elevation. In support of this, the concentration range of TPA found to inhibit PAF-induced $[Ca^{++}]_i$ elevation in this study correlates well with the concentrations found to activate PKC in intact platelets in other studies (Zavoico *et al.*1985) and indeed in this study it was found that 30 nM TPA caused a significant level of PKC activation as demonstrated by the phosphorylation of the 40-47 kDa substrate of PKC. Moreover, the inhibitory effect of TPA on $[Ca^{++}]_i$ elevation is prevented by pretreatment with the PKC inhibitor staurosporine, which further suggests that the effects observed with TPA are a consequence of PKC activation.

It is known however, that TPA possesses many different properties compared to endogenously produced sn-1,2-DAG (Bazzi and Nelsestuen, 1989). One difference is the longevity, whereas DAG is rapidly metabolized and activates PKC in a transitory fashion, phorbol esters are degraded slowly and cause a prolonged activation of PKC (Nishizuka, 1986). In addition, phorbol esters are more permeant than DAGs raising

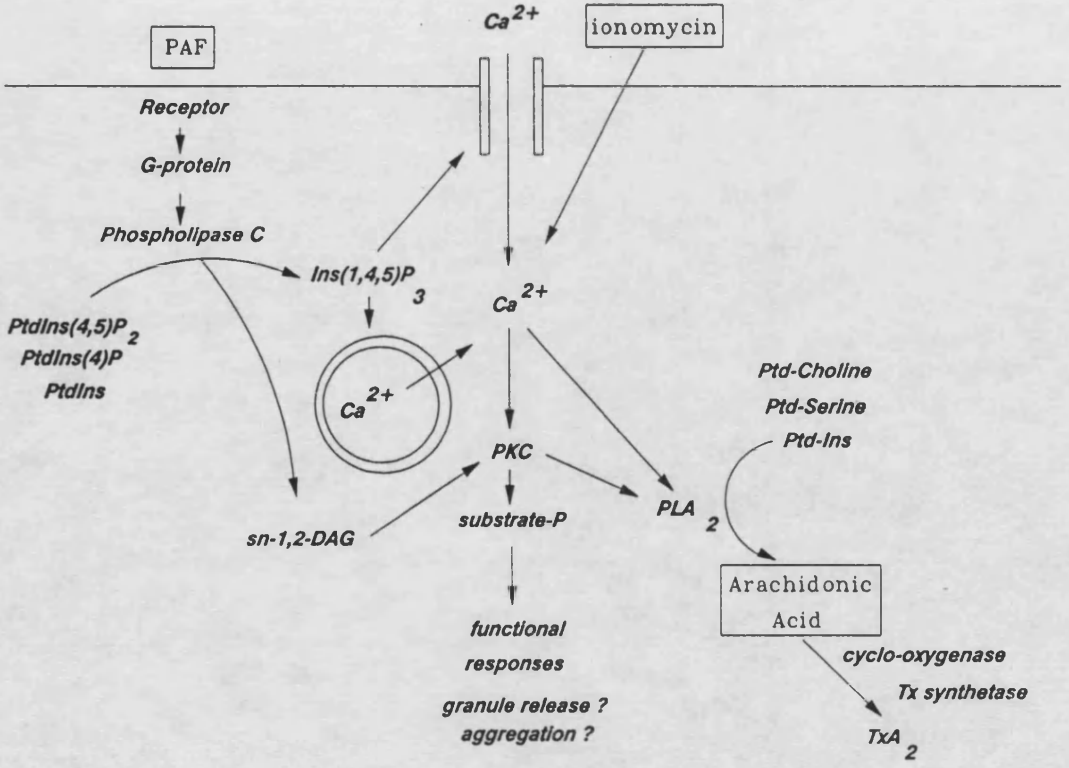

Fig 63 A schematic drawing of the different mechanisms of platelet activation by the three agents PAF, ionomycin and AA.

the possibility that phorbol esters may activate PKC at sites unavailable to DAG and therefore present different substrates to PKC. In order to examine whether TPA was a suitable substitute for DAG with which to investigate the role of PKC, the effect of PKC activation with a membrane permeant DAG, namely, 1,2-dihexanyl-sn-diacylglycerol (DHG) on PAF-induced $[Ca^{++}]_i$ elevation was investigated. Pretreating platelets with DHG, which possesses a greater similarity to physiologically relevant sn-1,2-DAG, caused the same inhibitory effect on $[Ca^{++}]_i$ elevation as TPA, which could also be prevented by pretreatment with staurosporine again suggesting a modulatory role for PKC in $[Ca^{++}]_i$ elevation.

It is unlikely that inhibition of $[Ca^{++}]_i$ elevation by activation of PKC in PAF stimulated platelets is due to TPA or membrane permeant DAG affecting binding of [^{3}H]PAF to platelets as concentrations of either TPA or DAG higher than used in this study, have no effect on [^{3}H]PAF binding to platelets (Valone and Johnson, 1987). In addition, phorbol esters have the same effect on $[Ca^{++}]_i$ elevation in platelets activated with a variety of agonists including thrombin, ADP, vasopressin and the Tx mimetic U46619 (Poll and Westwick, 1986a; Powling and Hardisty, 1986) which stimulate different receptors. Moreover, in platelets where the signal transduction pathway has been stimulated at a post-receptor level using NaF, in particular the AlF_4 moiety, which is thought to stimulate G-protein activation, pretreatment with phorbol ester had the same effect as in agonist-stimulated platelets indicating that modulation of $[Ca^{++}]_i$ by PKC is a post-receptor event (Poll *et al.*1986).

From studies where PKC has been exogenously activated using either phorbol esters, or membrane permeant DAGs, it has been suggested that PKC exerts a negative feedback pathway which by inhibiting the production of the signal molecules produced by PLC-induced PtdIns(4,5)P_2 hydrolysis (Zavoico *et al.*1985; Watson and Lapetina, 1985; Rittenhouse and Sasson, 1985) will subsequently inhibit Ca^{++} mobilization. As further evidence of a negative feedback role of activated PKC, increasing the endogenous concentration of DAG by the use of the DAG kinase inhibitor R44949 has also been shown to inhibit $[Ca^{++}]_i$ elevation in thrombin

stimulated platelets, suggesting endogenously activated PKC is also capable of exerting a negative feedback (De Chaffoy de Courcelles *et al.*1989).

In addition to inhibiting $[Ca^{++}]_i$ elevation, pretreatment of platelets with TPA also caused an inhibition of TxB_2 generation in PAF stimulated platelets. TxB_2 is the stable metabolite of TxA_2 which is generated from AA via the cyclo-oxygenase pathway and TxA_2 synthetase (Hamberg *et al.*1975). AA is produced from two sources, it is either released from membrane phospholipids such as phosphatidylcholine by the action of the Ca^{++} and phospholipid dependent enzyme PLA_2 (thought to be the major source of AA (Bishop and Bell, 1986)) or by the action of di- and monoacylglycerol lipases on 1-stearoyl-2-arachidonyldiacylglycerol produced from the hydrolysis of PtdIns(4,5)P_2 by PLC (believed to be a minor source of AA in platelets (Bell *et al.*1979; Mahaevappa and Holub, 1986)). Many platelet functional responses are dependent to some degree on $[Ca^{++}]_i$. Replacing $[Ca^{++}]_e$ with the Ca^{++} chelator EGTA caused an inhibition of $[Ca^{++}]_i$ elevation, TxB_2 generation and dense granule release, in platelets stimulated with a range of concentrations of PAF suggesting that these functional responses are dependent on $[Ca^{++}]_i$ elevation. It is possible that in PAF-stimulated platelets the inhibition of TxB_2 caused by the PKC activation may be a direct consequence of the inhibition of $[Ca^{++}]_i$ elevation, particularly as it is known that PLA_2 is a Ca^{++} dependent enzyme (Baron and Limbird, 1988). However, this may not be true for all agonists, as collagen has been shown to activate PLA_2 independently of changes in $[Ca^{++}]_i$ (Pollock *et al.*1986).

Other possibilities for the regulation of Tx generation by PKC also exist. There is now increasing evidence that PLA_2 activation may be directly coupled to membrane receptors by GTP-binding proteins (Silk *et al.*1989) therefore by-passing PLC activation and suggesting that not all PLA_2 activation is subsequent to PLC activation, but may be a separate event. Evidence for the presence of G-proteins which couple PLA_2 to receptors in platelets includes the finding that the dose response profiles of both pertussis toxin and GDPßS required to inhibit thrombin-induced AA release (and therefore PLA_2 activity) in permeabilized platelets were different from those required to inhibit DAG production (therefore PLC activity) suggesting a G-protein coupled to

PLA_2 which is pertussis toxin sensitive and may be distinct from the G-protein responsible for modulation of PLC (Kajiyama *et al.*1989; Nakashima *et al.*1987). In addition, PGI_2 has a differential ability to inhibit PLC activity compared to PLA_2 activity in thrombin activated platelets indicating these enzymes may be activated by separate mechanisms during thrombin stimulation (Crouch and Lapetina, 1988). It is possible therefore that in the same way as the G-protein(s) which couples the receptor to PLC may be regulated by PKC-induced phosphorylation, the G-protein which couples the receptor to PLA_2 may also be regulated by PKC.

As an alternative experimental approach the potent, although non-specific, protein kinase inhibitor staurosporine (Tamaoki *et al.*1986) was employed to investigate the role of endogenously activated PKC. Inhibition of PKC activity by staurosporine was confirmed by investigating its ability to inhibit PAF-induced PKC substrate phosphorylation. Staurosporine was found to cause a dose-dependent inhibition of 300 nM PAF-induced phosphorylation of the 40-47 kDa substrate of PKC with an IC_{50} of 500 nM. Other studies have also shown inhibition of phosphorylation of the 40-47 kDa PKC substrate over the same range of concentrations of staurosporine in platelets stimulated with different agonists (Watson *et al.*1987). In the original study of Tamaoki *et al.* (1986) the IC_{50} for the inhibition of PKC by staurosporine was determined as 2.7 nM. This value was obtained using isolated PKC in an *in vitro* assay where the effect of staurosporine on DAG-induced PKC-mediated histone phosphorylation was investigated. It is now known that staurosporine inhibits PKC activity by competing with ATP for its binding site on the catalytic subunit of PKC (Ruegg and Burgess, 1989). The concentration of $< 10\ \mu M$ ATP used in the *in vitro* assay of Tamaoki *et al.* is therefore much lower than the mM concentration of ATP in intact cells (Burt *et al.*1976) and would explain the much lower potency of staurosporine at inhibiting PKC in an intact cell preparation. The ATP binding site of protein kinase C has a high homology with the ATP binding site of other protein kinases (Ruegg and Burgess, 1989), therefore inhibitors which act by competing with ATP are likely to be only poorly selective at inhibiting protein kinase C and this is true for staurosporine. Indeed, staurosporine has previously been shown to inhibit

phosphorylation of the 20 kDa protein known to be the substrate of myosin light chain kinase (Watson *et al.*1987) which is phosphorylated in parallel with the 40-47 kDa protein upon agonist-receptor coupling in platelets (Kaibuchi *et al.*1983).

If endogenously activated PKC is exerting a negative feedback and inhibiting PLC activity it would be expected that removal of this inhibitory effect would result in the opposite effect. Pretreatment with a range of staurosporine known to inhibit phosphorylation of the 40-47 kDa substrate had no significant effect on the maximal elevation of $[Ca^{++}]_i$ induced by 3 nM PAF. However, in contrast to the effect of PKC activation with TPA, staurosporine caused a potentiation in TxB_2 generation, suggesting endogenously activated PKC is inhibiting TxB_2 generation and supporting a negative feedback role for endogenously activated PKC.

Earlier observations have demonstrated that elevation of $[Ca^{++}]_i$ using either an ionophore or a permeabilized platelet preparation in combination with PKC activation causes a synergistic increase in platelet activation (Mobley and Tai, 1985; Halenda *et al.*1989). These findings suggest that PKC has a positive role in platelet activation, requiring both PKC activation and $[Ca^{++}]_i$ elevation to elicit full platelet activation (Nishizuka, 1984). The results of this study confirm and extend these observations by demonstrating that whilst pre-incubation with the PKC activator TPA had no effect on maximal $[Ca^{++}]_i$ elevation induced by ionomycin, both 5-HT release and TxB_2 generation were significantly potentiated. A recent study demonstrated that the potentiation of AA release by phorbol esters in platelets was not due to inhibition of [^{3}H]AA uptake into platelets or incorporation into phospholipids (Banga *et al.*1991) but probably due to promoted release of AA. Staurosporine also had no effect on maximal $[Ca^{++}]_i$ elevation in ionomycin stimulated platelets, however in contrast to the effects of PKC activation both 5-HT release and TxB_2 generation were significantly inhibited. Therefore in ionomycin stimulated platelets activation of PKC potentiates functional responses whilst inhibition of PKC suppresses them. Ionomycin has been demonstrated to liberate AA directly (Rittenhouse, 1984; Halenda *et al.*1989), and in agreement with this inhibition of the cyclo-oxygenase pathway by indomethacin had no significant effect on either ionomycin induced $[Ca^{++}]_i$ elevation

or dense granule release. This would indicate that ionomycin induced release of AA and therefore products of the cyclo-oxygenase pathway, in particular TxA_2, were not influencing either of these processes and that ionomycin-induced platelet activation and AA-induced platelet activation can be thought of as separate mechanisms of platelet activation.

Several studies have used a combination of ionophores (to mimic $Ins(1,4,5)P_3$ production by increasing $[Ca^{++}]_i$) and either membrane permeant DAGs or phorbol esters (to mimic endogenously produced sn-1,2-DAG by activating PKC) and have suggested that using these agents a model is manifested which reproduces the effect of agonist-induced platelet activation (Kaibuchi *et al.*1983; Nishizuka, 1984). The results presented in this thesis demonstrate that the effects of PKC modulation in platelets stimulated with ionomycin differ greatly from those observed in agonist-stimulated platelets (figure 64), and make the use of ionophores together with PKC activators a poor model to use to investigate the role of endogenously activated PKC in a physiological system.

In ionomycin-stimulated platelets therefore $[Ca^{++}]_i$ elevation is not affected by PKC modulation but TxB_2 generation is affected. The finding that ionomycin is able to induce the generation of TxB_2 suggests that an increase in $[Ca^{++}]_i$ alone is able to directly activate PLA_2 and cause the release of AA from phospholipids. Both cyclo-oxygenase and TxA_2 synthetase are Ca^{++} independent enzymes and therefore an increase in $[Ca^{++}]_i$ would not be expected to increase the metabolism of AA to TxA_2. Phorbol ester and staurosporine respectively potentiate and inhibit TxB_2 generation in ionomycin stimulated platelets, but not by affecting $[Ca^{++}]_i$ nor by influencing PLC activity (Rittenhouse, 1984). Instead, it is possible that PKC directly regulates PLA_2 activity (as AA production is independent of PLC activation) or influences the activity of an intermediate protein responsible for the regulation of PLA_2 activity. Such an intermediate protein may be an annexin (previously termed lipocortins/calpactins) (Crumpton and Dedman, 1990; Russo-Marie, 1991).

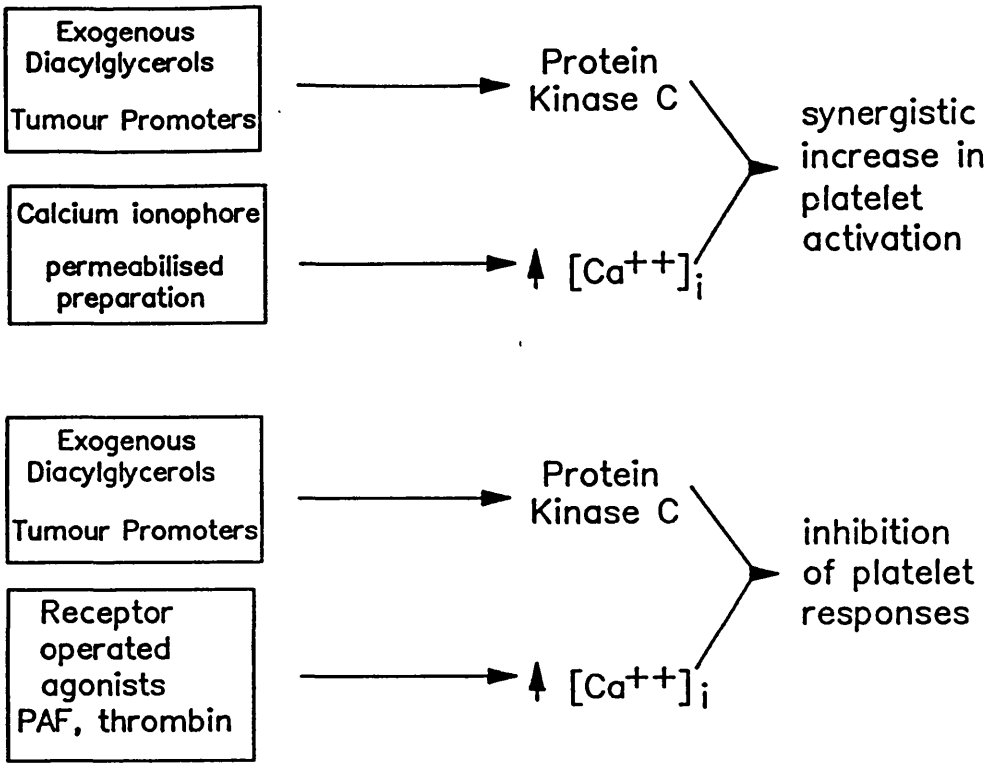

Fig 64 Flow diagram showing the differential effect of exogenous PKC activation in either agonist or ionomycin stimulated platelets.

Members of the annexin family are proteins which were thought to be endogenous inhibitors of PLA_2 (Parente *et al.*1984; Wallner *et al.*1986) although this is now controversial (Davidson and Dennis, 1989). Several annexins are substrates for PKC (at least *in vitro*) (Coméra *et al.*1989; Khanna *et al.*1986) and it has been suggested that phosphorylated annexin does not inhibit PLA_2 (Hirata, 1981). Indeed, in this study evidence is presented, for the presence of several members of the annexin family in platelets (discussed later).

Another possibility worthy of consideration is the finding that activation of PKC with phorbol esters or membrane permeant DAGs increases cytosolic pH by stimulating Na^+/H^+ exchange (Siffert and Akkerman, 1988b), and that addition of a Ca^{++} ionophore to platelets also triggers a proton efflux which has all the characteristics of Na^+/H^+ exchange (Siffert and Akkerman, 1988a). This Na^+/H^+ exchange results in alkalinization of the cytosol which favours PLA_2 activity (Baron and Limbird, 1988). However, inhibition of the Na^+/H^+ antiporter by dimethylamiloride had no effect on the potentiation of ionophore-induced AA release by phorbol ester in human platelets suggesting that phorbol ester/ionophore synergism is not mediated by the Na^+/H^+ antiporter (Halenda *et al.*1989). Moreover, the effect of alkalinization by the Na^+/H^+ antiporter on PLA_2 activity in agonist-stimulated platelets is more doubtful, as TxB_2 generation and therefore PLA_2 activation is inhibited by phorbol ester treatment, suggesting that the potentiating effect of alkalinization is overcome by some more powerful factor.

It is unlikely that the cyclo-oxygenase pathway or Tx synthetase are the sites of PKC modulation, as neither phorbol ester nor staurosporine modified AA-induced TxB_2 generation. However similar to PAF, TxA_2-induced $[Ca^{++}]_i$ elevation was dose-dependently inhibited by pretreatment with TPA, whilst staurosporine had no significant effect on maximal $[Ca^{++}]_i$ elevation.

PKC modulates dense granule release since activation and inhibition respectively potentiate and inhibit dense granule release in ionomycin-stimulated platelets. Inhibition of the cyclo-oxygenase pathway with indomethacin had no effect on the TPA-potentiated dense granule release in ionomycin stimulated platelets indicating

that the potentiation was not due to the parallel enhanced generation of TxA_2. In PAF-stimulated platelets TPA had no significant effect on 5-HT release, whilst in comparison to its effect in ionomycin stimulated platelets, staurosporine caused only a weak inhibition of 5-HT release. A good correlation is thought to exist between the phosphorylation state of the major 40-47 kDa protein substrate of PKC and dense granule release although a cause-effect relationship has not yet been demonstrated (Rink *et al.*1983). There is also some evidence that phosphorylation of MLC (20 kDa protein) is also correlated with dense granule release (Lyons and Shaw, 1980) and that phosphorylation of the 20 kDa protein may be a pre-requisite for the initiation of receptor-mediated dense granule secretion, although this correlation has also been disputed (Daniel *et al.*1984). Staurosporine can inhibit MLCKase activity (Watson *et al.*1987), therefore the inhibitory effect of staurosporine on dense granule release in this study may also be attributed to its inhibition of MLCKase. However, in this study using fura-2 loaded platelets and in previous studies which used quin-2 loaded platelets, activation of PKC with TPA was found to induce dense granule release and platelet aggregation despite no detectable increase in $[Ca^{++}]_i$ (Rink *et al.*1983; Yamada *et al.*1987) and both could be inhibited by non-selective PKC inhibitors (Yamada *et al.*1987). This would indicate a role for PKC in dense granule release independent of MLCKase activation, as MLCKase is Ca^{++} dependent (Hallam *et al.*1985). To add confusion however, in studies using the Ca^{++} probe aequorin to monitor $[Ca^{++}]_i$ levels, a transient elevation of $[Ca^{++}]_i$ was detected upon phorbol ester treatment (Salzman *et al.*1985). It is possible that the conflicting findings for the different Ca^{++} probes may be due to either the Ca^{++} buffering capacity of fura-2 and quin-2 masking the $[Ca^{++}]_i$ elevation induced by PKC activation, or that aequorin is able to monitor changes in $[Ca^{++}]_i$ in compartments not available to fura-2 or quin-2.

The findings in this study indicate that dense granule release, as opposed to TxB_2 generation may have a closer affinity to the phosphorylation state of PKC substrates. The finding that inhibition of PKC had the same effect on PAF-induced as on ionomycin-induced dense granule release makes it unlikely that modulation of PLC by

PKC is of primary importance in this particular functional response. However, staurosporine is a weaker inhibitor of PAF-induced (IC_{50} 800 nM) than of ionomycin-induced (IC_{50} 150 nM) dense granule release indicating that perhaps PKC has a less important role in modulating agonist-induced than ionomycin-induced dense granule release.

PAF itself has been described as the most active and specific inhibitor of PAF-induced platelet activation (Chignard *et al.*1985). Early work described how PAF-stimulated rabbit platelets did not respond to a second challenge with PAF, but would still respond to other agonists (Keraly and Benveniste, 1982). The mechanism of desensitization of PAF to a second challenge is unknown, but it is possible that PKC may be involved as activation of PKC and subsequent protein phosphorylation causes an inhibition of many functional responses. Activation of PKC has been demonstrated to exert a negative feedback inhibiting PLC activity and this may be due to uncoupling the receptor from the G-protein which activates PLC (therefore making the response specific for a particular receptor). Challenging platelets with the first dose of PAF would therefore endogenously activate PKC and cause a negative feedback inhibiting either directly or indirectly the action of PLC and preventing activation of platelets by challenge with a second dose of PAF. It is possible that removal of this negative feedback by PKC may also prevent desensitization to a second PAF stimulation. It has been demonstrated in this study that staurosporine is able to inhibit PKC activation induced by PAF and is also able to abrogate the effect of PKC activation by TPA in PAF stimulated platelets. Therefore it is possible that if desensitization was due to inhibition of an early portion of the signal transduction pathway caused by PKC activation, that inhibition of PKC could block desensitization. Stimulation of platelets with a range of concentrations of PAF caused a dose dependent elevation of $[Ca^{++}]_i$ and in agreement with previous studies, a second dose of the same concentration of PAF produced either a much smaller increase in $[Ca^{++}]_i$ (with the lower concentrations of PAF), or no elevation in $[Ca^{++}]_i$ (with the higher concentrations of PAF) indicating desensitization of PAF receptors to PAF-stimulation. However, inhibition of PKC by pretreatment with staurosporine did not remove the

desensitization of the PAF receptor, inferring that PKC activation is not likely to be the mechanism underlying the desensitization process. This is in agreement with findings which have since been published by Morrison *etal* (Morrison *et al.*1989).

The results of this study suggest that PKC has a modulatory role in both PAF and ionomycin-activated platelets, but that the sites of modulation are different. The results infer that the phosphorylation of substrate(s) by PKC has a more important influence on platelet functional responses in ionomycin stimulated platelets where cytosolic Ca^{++} levels are not affected by PKC activation or inhibition and where modulation of PLC activity (upon TxA_2-receptor coupling) is not likely to be of primary importance. In PAF-stimulated platelets however, the effect of PKC modulation on PLC activity and on $[Ca^{++}]_i$ elevation are probably more important factors in modulating TxB_2 generation, but other factors as discussed may also play a role. The different effects of PKC modulation on PAF-induced TxB_2 generation and dense granule release suggest a different relationship between PKC and dense granule release than between PKC and TxB_2 generation in PAF-stimulated platelets.

4.2.2 The relationship between signal molecule elevation and the influence of PKC on production of signal molecules in PAF-stimulated platelets

The relationship between the three putative signal molecules, namely $Ins(1,4,5)P_3$, DAG and $[Ca^{++}]_i$ has been examined in PAF-stimulated rabbit platelets and in addition the effects exerted by inhibition of PKC with staurosporine have also been determined.

A very rapid and transient elevation of all three signal molecules was induced by the addition of a high concentration of PAF. The concentration of $Ins(1,4,5)P_3$ reached a peak at 2 s after PAF addition, which preceded the peak concentration of $[Ca^{++}]_i$ by 5 s. In addition, the peak concentration of $Ins(1,4,5)_3$ was 45 pmol/10^9 platelets, which is equivalent to 4.5 μM (assuming that 10^9 platelets have a cytosolic volume of 10 μl (Rittenhouse and Sasson, 1985)), close to the 5 μM $Ins(1,4,5)P_3$ that is required for maximum release of $^{45}Ca^{2+}$ from permeabilized human platelets (Brass and Joseph, 1985) and membrane vesicles (O'Rourke *et al.*1985). Thus our

observations in intact platelets provide good support for the proposal that Ins(1,4,5)P_3 is responsible for mobilizing intracellular Ca^{2+} (Berridge and Irvine, 1989).

The time course and the amount of DAG formed, compared with Ins(1,4,5)P_3 suggests that PtdIns(4,5)P_2 hydrolysis may not be the only source of DAG. Possible additional sources include the hydrolysis of other phosphoinositides (Rittenhouse-Simmons, 1979; Majerus *et al.*1985) or the action of PLD on phosphatidylcholine (Rubin, 1988; Huang *et al.*1991). Whatever the source of DAG, the 3 fold increase over basal levels reported in the present study with PAF-stimulated rabbit platelets corresponds well to the 2-3 fold DAG elevation found in thrombin-stimulated human platelets, when the same assay for DAG was used (Preiss *et al.*1986). The parallel inhibition of PAF-induced elevations of Ins(1,4,5)P_3 and DAG by WEB 2086 (Casals-Stenzel, 1987) indicated that these events are coupled to a single class of PAF receptor.

The higher concentrations of PAF (30 and 300 nM) produced dose-related increases in the amounts of $[Ca^{++}]_i$, Ins(1,4,5)P_3, DAG, 40 kDa phosphorylation and granule release. However, sub-maximal concentrations of PAF (0.3 and 3 nM), which caused considerable dense-granule release and elevation of $[Ca^{++}]_i$, failed to induce a detectable increase over basal levels in the concentration of either Ins(1,4,5)P_3 or DAG. Thus, in platelets activated with sub-maximal levels of PAF, neither the Ins(1,4,5)P_3-dependent mobilization of Ca^{++} from intracellular stores nor the DAG-dependent activation of PKC would be expected. The lack of $[Ca^{++}]_i$ elevation in the absence of $[Ca^{++}]_e$ at low levels of PAF, together with the decreased phosphorylation of PKC substrate with sub-maximal levels of PAF, supports this.

These findings have several implications. Firstly, at higher concentrations of PAF, there is a good correlation between products of Ptd(4,5)InsP_2 hydrolysis and platelet activation, but at the lower concentrations of PAF, platelet activation, including Ca^{++} mobilization, occurs independently of PtdIns(4,5)P_2 hydrolysis. Indeed, in other cell types a lack of correlation between PKC activation and functional response, or Ins(1,4,5)P_3 elevation and $[Ca^{++}]_i$ elevation, has been found. A lack of correlation between DAG accumulation and superoxide-anion production after stimulation of

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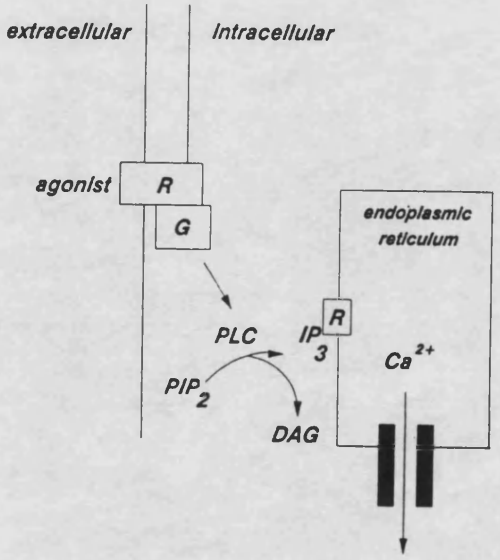
neutrophils with either concanavalin A (Rider and Niedel, 1987) or N-formylmethionyl-leucyl-phenylalanine has been observed (Koenderman *et al.*1989). In vascular smooth-muscle cells stimulated with PAF, the time to peak $[Ca^{++}]_i$ elevation is reached before any elevation in $Ins(1,4,5)P_3$, and it has been suggested that other mechanisms are responsible for regulating PAF-induced Ca^{++} mobilization, namely a receptor-operated Ca^{++} channel independent of phosphatidylinositol polyphosphate hydrolysis (Schwertschlag and Whorton, 1988). Similarly in ADP stimulated platelets Ca^{++} entry was found to clearly precede internal discharge, and it was suggested that receptor occupancy is closely coupled to the opening of plasma-membrane bivalent-cation channels (Sage *et al.*1989). Furthermore, in hepatocytes the formation of $Ins(1,4,5)P_3$ has been found to require a higher concentration of receptor-operated hormone than does $[Ca^{++}]_i$ elevation (Lynch *et al.*1985). This finding has been explained as either $Ins(1,4,5)_3$ elevation and $[Ca^{++}]_i$ elevation not being causally related, or by the presence of separate receptors for agonist that mobilize Ca^{++}. Moreover, PAF has been shown to increase $[Ca^{++}]_i$ in neutrophils where PLC activation and therefore $Ins(1,4,5)P_3$ production, have been inhibited by pertussis toxin (Naccache *et al.*1985).

The reduced elevation of $[Ca^{++}]_i$ by PAF upon replacement of $[Ca^{++}]_e$ with the Ca^{++} chelator EGTA demonstrated that $[Ca^{++}]_i$ elevation in PAF-stimulated platelets was due to two components. One component is the mobilization of Ca^{++} from intracellular stores, which in the absence of $[Ca^{++}]_e$ was found to be transient and quickly returned to basal levels. Indeed, in this study $[Ca^{++}]_i$ elevation was demonstrated to follow $Ins(1,4,5)P_3$ production therefore supporting the role of $Ins(1,4,5)P_3$ in the mobilization of Ca^{++} from internal pools. Moreover, during the course of this study the cloning and sequencing the $Ins(1,4,5)P_3$ receptor (Furuichi *et al.*1989; Mignery *et al.*1989) together with the revealed homologies with the Ca^{++} releasing channel of skeletal muscle known as the ryanodine receptor (Fill and Coronado, 1988) were major advances in establishing the Ca^{++} mobilizing role of $Ins(1,4,5)P_3$. In addition the development of synthetic and stable analogues of $Ins(1,4,5)P_3$ including $Ins(1,4,5)P[S]_3$ which have been demonstrated to mobilize

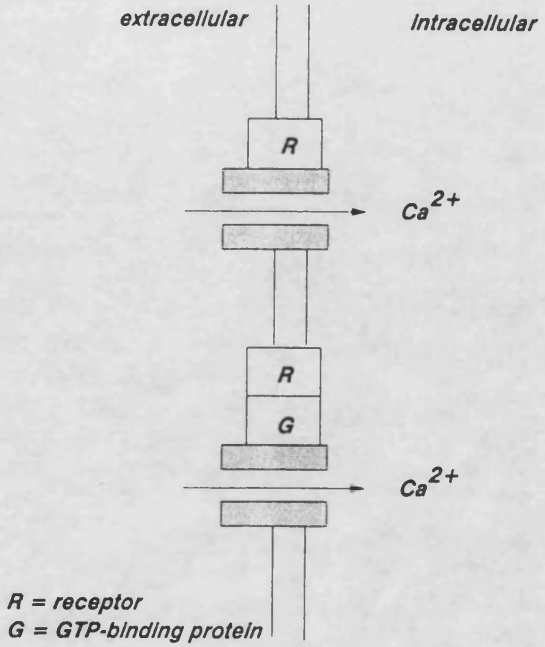
intracellular Ca^{++} have also substantiated the role of $Ins(1,4,5)P_3$ in Ca^{++} mobilization and will also facilitate in separating the functions of $Ins(1,4,5)P_3$ from those which require the presence of its metabolic products (Taylor *et al.*1989). However, a combination of the small size of platelets, together with the necessity for a permeabilized cell preparation in order to use the synthetic analogues of $Ins(1,3,4)P_3$ has resulted in a lack of studies so far in this field in platelets. The other component of $[Ca^{++}]_i$ elevation in PAF-stimulated platelets is Ca^{++} influx across the plasma membrane, which was responsible for the sustained duration of $[Ca^{++}]_i$ elevation. It is known that platelets lack voltage-dependent Ca^{++} channels such as those described in nerve and muscle (Hallam and Rink, 1985a). The mechanism(s) of Ca^{++} influx across the plasma membrane in agonist-stimulated platelets is unknown, but in other cell types there is evidence that Ca^{++} influx may be through receptor operated channels (ROCs) which are either tightly coupled directly to the receptor or which are regulated by receptor-coupled G-proteins (Rink, 1990). An additional mechanism of Ca^{++} influx may be through Ca^{++} channels which are modulated indirectly through some internal diffusible messenger (possibly a combination of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$) and which are termed second messenger operated channels (SMOC) (Berridge and Irvine, 1989; Rink, 1990). Several models have been suggested for Ca^{++} influx through SMOC(s) including a capacitative model where Ca^{++} enters into an intracellular pool before entering the cytosol, with the Ca^{++} concentration in the pool somehow regulating Ca^{++} entry, reviewed in (Berridge and Irvine, 1989; Irvine, 1990). Only part of the intracellular Ca^{++} pool appears to be $Ins(1,4,5)P_3$-sensitive (Ghosh *et al.*1989) and it is thought that $Ins(1,3,4,5)P_4$ may regulate the transfer between intracellular stores and indeed, if some of these pools regulate Ca^{++} entry across the plasma membrane, this may explain the requirement for $Ins(1,3,4,5)P_4$ as described in the 'quantal' model of Irvine (Irvine, 1990). The key aspect of this model however is the possession on the $Ins(1,4,5)P_3$ receptor of an intraluminal allosteric Ca^{++} binding site which interacts with the plasma membrane (Irvine, 1990). Possible evidence for the presence of SMOCs in platelets includes a study demonstrating that an increase in cAMP levels with forskolin, which inhibited

PtdIns(4,5)P_2 hydrolysis and therefore Ins(1,4,5)P_3 elevation, also inhibited the second phase of ADP-induced $[Ca^{++}]_i$ elevation observed in the presence of $[Ca^{++}]_e$ and in addition inhibited the second phase ADP-evoked Mn^{++} quench, indicating that Ca^{++} entry may be dependent either directly or indirectly on second messenger production, although not dismissing the possibility that influx maybe due to the state of filling of the intracellular stores (Sage *et al.*1990). It is believed that SMOC(s) are only responsible for a small influx of Ca^{++} but that they drive Ca^{++} oscillations in many cells (Berridge and Irvine, 1989). In most studies, (including this study) which have investigated the dynamic changes in $[Ca^{++}]_i$ uponagonist stimulation, cell populations are used, however, if $[Ca^{++}]_i$ is oscillating asynchronously in the cells, Ca^{++} oscillations of individual cells would be masked. Figure 65 demonstrates some of the possible mechanisms responsible for elevating $[Ca^{++}]_i$.

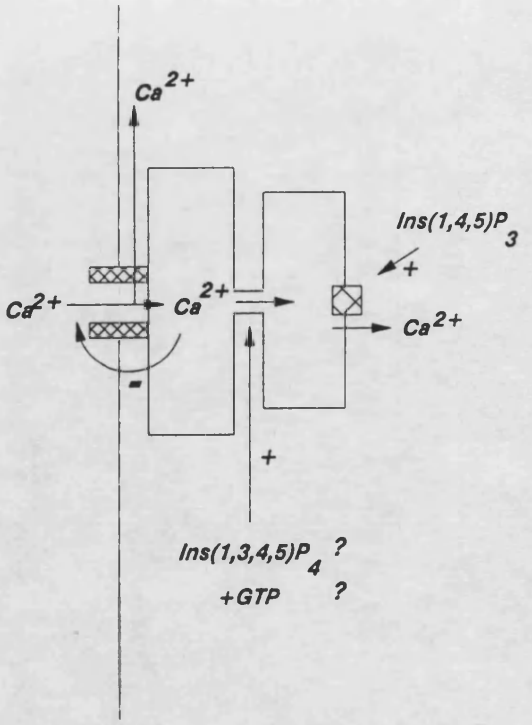
In this study, at the higher concentrations of PAF, $[Ca^{++}]_i$ elevation was found to be more dependent on Ca^{++} influx than Ca^{++} mobilization, with mobilization of Ca^{++} from the intracellular stores reaching a plateau, whilst $[Ca^{++}]_i$ continued to rise with increasing concentrations of PAF. Sub-maximal concentrations of PAF induced $[Ca^{++}]_i$ elevation as a result of a combination of both Ca^{++} mobilization and influx, whilst at low concentrations of PAF where no PLC activity was detected, $[Ca^{++}]_i$ elevation was found to be totally dependent on Ca^{++} influx. This finding supports the presence of ROCs for Ca^{++} coupled to the PAF-receptor independent of elevation of Ins(1,4,5)P_3 or its metabolites. One possible explanation for this could be the presence of two binding sites for PAF, with one being a high affinity binding site coupled to the ROCs, however, from the literature it appears that there is only one binding site which has an affinity for PAF in the nM range on rabbit platelets, which must therefore also be coupled to PLC (Hwang *et al.*1986), this finding is also true of human platelets (Valone *et al.*1982). However, there is evidence for the availability of multiple conformational states of a single type of PAF receptor on rabbit platelets (Hwang *et al.*1989). There is also evidence that ADP receptors in platelets are linked to ROCs.



IP_3 induced calcium mobilization



Receptor operated calcium channels



Extended capacitative model. One proposed mechanism for a second messenger operated calcium channel which includes a role for $Ins(1,3,4,5)P_4$ and GTP

Fig 65 Mechanisms of $[Ca^{++}]_i$ elevation.

In studies where Mn^{++} was used as a surrogate for Ca^{++} ADP-induced platelets Ca^{++} entry clearly preceded Ca^{++} discharge from the internal stores suggesting the presence of ROCs for Ca^{++} entry linked to the ADP receptor (Sage *et al.*1990; Sage *et al.*1989). These findings correlate well with studies which demonstrate that ADP is able to activate single channels permeable to Ba^{++} in intact human platelets (Mahaut-Smith *et al.*1990). Moreover, it is interesting that the characteristics of Ca^{++} influx in platelets stimulated with PAF and ADP were found to be similar to each other both temporally and in their longevity but different to those induced by either thrombin or collagen (Alonso *et al.*1989).

To determine the role of PKC in the regulation of early post-receptor events, the potent, although non-specific, PKC inhibitor staurosporine was used (Tamaoki *et al.*1986; Watson *et al.*1987). A concentration of 1 μM staurosporine was selected, as this concentration inhibited by at least 70% 300 nM PAF induced PKC substrate phosphorylation. Both DAG and Ins(1,4,5)P_3 production induced by 300 nM PAF were significantly potentiated by PKC inhibition; however, there was no effect on DAG or Ins(1,4,5)P_3 levels in platelets stimulated with 3 nM PAF.

Conversely, pretreatment of platelets with the PKC activator TPA caused an inhibition of both DAG and Ins(1,4,5)P_3 in platelets stimulated with either 3 or 300 nM PAF. This finding agrees with previous studies where pretreatment with phorbol esters or membrane permeant DAG resulted in an increase in the levels of PtdIns(4,5)P_2 and PtdIns(4)P in agonist stimulated platelets predicting an inhibition of their hydrolysis (Watson and Lapetina, 1985; Zavoico *et al.*1985). The inhibitory effects of pre-activation of PKC on platelet functional responses demonstrated in this and other studies in both agonist and fluoride stimulation platelets (Poll and Westwick, 1986a; Poll *et al.*1986; Krishnamurthi *et al.*1986) lends increasing evidence to the theory that, when activated, PKC exerts a negative feedback inhibiting generation of both DAG and Ins(1,4,5)P_3 at the level of production (at either PLC or a regulating G-protein) (Watson and Lapetina, 1985; Zavoico *et al.*1985).

The findings of this study suggest that in our system, where PKC is activated by higher concentrations of PAF, DAG and Ins(1,4,5)P_3 levels are depressed by negative feedback. However, when PKC is inhibited the negative feedback is removed and a full elevation of DAG and Ins(1,4,5)P_3 induced by 300 nM PAF stimulation results. At lower concentrations of PAF no negative feedback would be exerted by PKC, and therefore the inhibition of PKC would, as observed, have no effect. Although these findings suggest that PKC is exerting its effect on the production of Ins(1,4,5)P_3 and DAG, as they are both formed from the hydrolysis of Ptd(4,5)InsP_2 by PLC, the findings do not dismiss the possibility that PKC may also have a role in the removal of Ins(1,4,5)P_3 and DAG. Indeed King & Rittenhouse have demonstrated that inhibition of PKC by staurosporine decreases inositol 5-phosphomonoesterase activity and is therefore at least partially responsible for the potentiation in Ins(1,4,5)P_3 in human platelets exposed to thrombin (King and Rittenhouse, 1989). However, Watson and colleagues have demonstrated that 1 μM staurosporine has no effect on the conversion of the membrane permeant DAG, namely DC8 to PA, indicating inhibition of PKC does not modulate DAG kinase activation (Watson *et al.*1987). In addition in our system the potentiation of Ins(1,4,5)P_3 by staurosporine is very transient, which would not be expected if the metabolism of Ins(1,4,5)P_3 was being significantly inhibited by removal of PKC activity with staurosporine. Another interesting point from these findings is that the activator of PKC namely DAG would appear to be self-regulating.

These findings are however in contrast to those presented by Watson *et al* (Watson *et al.*1987) where, in platelets stimulated with thrombin, pretreatment with 1 μM staurosporine had no effect on either PA formation (as a marker of DAG elevation; although PA may also be directly produced by the action of PLD on phosphatidylcholine (Huang *et al.*1991)) or inositol phosphate levels. From the findings of their study they suggested that PKC activation did not exert a negative feedback over PLC. An important reason for this difference in the two studies is probably that the time chosen to measure PA and inositol phosphate levels after thrombin stimulation in their study (60 s) may have been too late and that, at 60 s

post-thrombin, levels of PA and inositol phosphates may have already declined. Indeed at this time point in our system both DAG and Ins(1,4,5)P_3 levels had already reached near basal levels. Moreover, in our system the potentiation of Ins(1,4,5)P_3 by staurosporine was only transient, and at 60 s post-PAF there was no significant difference in Ins(1,4,5)P_3 levels in platelets either pretreated or not pretreated with staurosporine, and in this respect our findings would agree with those of Watson *etal* (Watson *et al.*1987).

An attempt was also made to correlate the effect of PKC inhibition and activation on generation of DAG or Ins(1,4,5)P_3 with the effect on the functional responses of $[Ca^{++}]_i$ elevation and TxB_2 generation. Activation of PKC inhibited Ins(1,4,5)P_3 and DAG production which would account for the inhibition of $[Ca^{++}]_i$ mobilization induced by PKC activation in PAF stimulated platelets described earlier in this study. In addition activated PKC has also been demonstrated to increase the rate of Ins(1,4,5)P_3 dephosphorylation to inactive Ins(1,4)P_2 by phosphorylating 5'-phosphomonoesterase (Molina y Vedia and Lapetina, 1986; Connolly *et al.*1986) again reducing Ca^{++} mobilization from the intracellular stores. Influx of Ca^{++} across the plasma membrane via signal-molecule-operated Ca^{++} channels may also be regulated by Ins(1,4,5)P_3, working in concert with one of its phosphorylated products, namely Ins(1,3,4,5)P_4 (Irvine, 1990) therefore reduction of Ins(1,4,5)P_3 by inhibition of PLC may also inhibit second messenger operated Ca^{++} channels. Moreover, activation of PKC may also stimulate removal of $[Ca^{++}]_i$ by activating the Ca^{++}-ATPase on the membrane of the storage sites and also the Na^+/Ca^{++} exchange system on the plasma membrane (Nishizuka, 1986). A study using Mn^{2+} as a surrogate for Ca^{2+} (Valone and Johnson, 1987) demonstrated that PAF-induced Ca^{++} mobilization was more sensitive to the inhibitory effects of TPA than Mn^{2+} influx, perhaps indicating different mechanisms of modulation of the two pathways of $[Ca^{++}]_i$ elevation by PKC.

Staurosporine had no significant effect on either 3 nM or 300 nM PAF-induced peak $[Ca^{++}]_i$ elevation, despite a potentiation in levels of Ins(1,4,5)P_3 upon stimulation with 300 nM PAF. This could be due to the limits of detection of $[Ca^{++}]_i$ elevation

being reached after stimulation with 300 nM PAF and therefore any further increase in $[Ca^{++}]_i$ being indistinguishable, or that maximal intracellular Ca^{2+} mobilization had already been achieved. Indeed, in support of the latter theory, staurosporine had no effect on the maximal mobilization of $[Ca^{++}]_i$ from intracellular stores in platelets stimulated with 300 nM PAF in absence of $[Ca^{++}]_e$ (where $[Ca^{++}]_i$ had been replaced with the Ca^{++} chelator EGTA). There was no increase in $Ins(1,4,5)P_3$ at 3 nM PAF however, and although it would have been possible to detect a potentiation in $[Ca^{++}]_i$ by staurosporine at this concentration of PAF, no significant increase was observed. However, when investigating the $t_{1/2}$ of the decay of $[Ca^{++}]_i$ it was found that PKC inhibition produced a 200% increase in the $t_{1/2}$ of elevated $[Ca^{++}]_i$ in 300 nM PAF-stimulated platelets but only a 30% increase in $t_{1/2}$ in platelets stimulated with 3 nM PAF. These findings are in agreement with the work of Watson & Hambleton (Watson and Hambleton, 1989), where an increase in $t_{1/2}$ of $[Ca^{++}]_i$ decay was observed in thrombin-stimulated platelets pretreated with 1 μM staurosporine, despite no evidence of a potentiation of $Ins(1,4,5)P_3$ (Watson *et al.* 1987).

At this point it is important to consider the effects of staurosporine on PAF-induced platelet aggregation. Pretreatment of platelets with staurosporine caused a dose-dependent, although only partial, inhibition of PAF-induced aggregation, without affecting shape change, which is in agreement with other studies (Watson and Hambleton, 1989; Siess and Lapetina, 1989). Indeed it has been suggested that platelets possess two pathways of platelet aggregation, a phosphorylation dependent one which can be inhibited by staurosporine, and a phosphorylation independent pathway which is induced by $[Ca^{++}]_i$ mobilization and which staurosporine is not able to inhibit (Watson and Hambleton, 1989). It has previously been demonstrated that in platelets loaded with fura-2, aggregation of platelets may interfere with and reduce the fluorescence signal and therefore the calculated $[Ca^{++}]_i$ (Cobbold and Rink, 1987). It is therefore possible that the longer duration of $[Ca^{++}]_i$ elevation observed in platelets pretreated with staurosporine may be due to the partial inhibition of platelet aggregation. To examine this possibility, the duration of $[Ca^{++}]_i$ elevation either in the presence or absence of staurosporine was determined in platelets which were

prevented from aggregating. Prevention of platelet aggregation was achieved by (a) terminating platelet stirring 10 s after the addition of PAF or (b) pretreating platelets with 870 μM of the tetrapeptide ARG GLY ASP SER preventing fibrinogen binding to GPIIb/IIIa receptors which is a prerequisite for aggregation (Plow *et al.*1985). The absence of platelet aggregation was found to have no effect on the $t_{1/2}$ of the decay of $[Ca^{++}]$ indicating a real role for PKC in platelet Ca^{++} homeostasis.

Indeed, the potentiation of the $t_{1/2}$ by inhibiting PKC, supports earlier findings (Poll and Westwick, 1986a; Pollock *et al.*1987) where activation of PKC was found to increase the rate of decline of $[Ca^{++}]_i$ to basal levels in agonist stimulated platelets. These findings further suggests that the role of PKC in Ca^{++} homeostasis may be in the restoration of the elevated $[Ca^{++}]_i$ to basal levels. Whether the increase in the duration of elevated $[Ca^{++}]_i$ by staurosporine is due to an increased duration of Ca^{++} influx or mobilization, or a decrease in Ca^{++} efflux or resequestration into the intracellular Ca^{++} stores is however, not known.

The findings in this study are also in agreement with those of King & Rittenhouse (King and Rittenhouse, 1989), in respect of PKC inhibition potentiating $Ins(1,4,5)P_3$ levels, although conversely they found that this caused an increase in $[Ca^{++}]_i$ elevation. In their study they demonstrated that staurosporine also inhibited phosphorylation of MLC, however to determine the role of MLCKase in potentiating $Ins(1,4,5)P_3$ levels, they inhibited MLCKase using ML-7 and demonstrated that this had no effect on the potentiation of $Ins(1,4,5)P_3$. More importantly however, they found that 10 μM staurosporine caused no potentiation in thrombin-induced DAG levels. This is interesting because Bishop *et al* (Bishop *et al.*1990) found that, with a high concentration of the PKC inhibitor sphingosine, the DAG elevation induced by thrombin was abolished, but at lower concentrations of sphingosine the DAG level was potentiated, and they attributed the inhibition by sphingosine to an inhibition of proximal portions of the signal-transduction pathway at this higher concentration of the inhibitor. Therefore it may be that the concentration of 10 μM staurosporine used in the study by King & Rittenhouse (1989), which is 10 fold greater than used

in this study, has masked any potentiatory response which may have been observed at a lower concentration of staurosporine.

The effect of staurosporine on PAF-induced TxB_2 generation was also examined. At lower concentrations of PAF (0.3-3 nM), only trace amounts of TxB_2 were detected, but at higher PAF concentrations (30 nM and 300 nM) TxB_2 was dose-dependently increased, and this increase was potentiated by inhibition of PKC. In this and other studies it has been demonstrated that in either agonist or fluoride stimulated platelets activation of PKC causes an inhibition of TxB_2 generation (Poll *et al.*1986). Inhibition of PKC, and therefore inhibition of the negative feedback exerted by endogenously activated PKC, would indeed predict a potentiated TxB_2 generation.

The increase in TxB_2 induced by PKC inhibition may be attributed to a number of events. Firstly, PLA_2 is Ca^{++}-dependent, and it may be that the sustained duration of elevation of $[Ca^{++}]_i$ induced by PKC inhibition is increasing the activation of this enzyme and thus causing the observed potentiation in TxB_2. Alternatively, the increased production of DAG could provide, via DAG lipase, increased levels of AA and ultimately TxB_2 production (conversely, the use of indomethacin provided evidence that the increase in TxA_2 was not responsible for the increase in DAG production). Finally it is also possible that PKC is exerting a direct regulatory effect on PLA_2 or a closely coupled GTP-binding protein (Silk *et al.*1989) or that it is affecting TxB_2 production by modulating the activity of annexins which may be responsible for inhibiting PLA_2 activity (Touqui *et al.*1986).

Several conclusions can be drawn from the present work. The relationship between the concentrations of the signal molecules and platelet functional responses is very dependent on the dose of PAF employed. In high-dose PAF-activated platelets, the elevation of $Ins(1,4,5)P_3$ clearly precedes the $[Ca^{++}]_i$ elevation, supporting the proposal that $Ins(1,4,5)P_3$ is responsible for this mobilization of intracellular Ca^{++} stores. In contrast, in low-dose PAF-activated platelets, elevation of $[Ca^{++}]_i$ and granule release occurs in the absence of detectable increases in the concentration of $Ins(1,4,5)P_3$ or DAG, as well as the absence of Ca^{++} mobilization from intracellular stores or PKC-substrate phosphorylation.

These results suggest that in platelet activation induced by low concentrations of agonist, mechanisms other than PtdIns(4,5)P_2 hydrolysis are utilized for platelet activation. The existence of these separate mechanisms is supported by the experiments with staurosporine, which result in a marked potentiation of DAG, Ins(1,4,5)P_3 and TxB_2 production as well as a protracted Ca^{++} signal induced to a much greater extent by the higher doses of PAF. Finally, the results demonstrate that endogenously activated PKC operates as a powerful negative feedback regulator of signal molecules (figure 66).

During the course of this study it has become clear that rather than being a single enzyme (Inoue *et al.*1977) PKC is infact a family of isoenzymes (Nishizuka, 1989) of which at least 7 namely, α, β_1, β_2, τ, δ, ϵ and theca are now known to exist (Table 5) and indeed several additional undefined species may also exist. PKC isoenzymes consist of a catalytic (carboxy-terminal half) and a regulatory domain (amino-terminal half). The catalytic domain contains sequences, including the ATP binding site, which are similar to other proteins kinases (Nishizuka, 1989). The regulatory domain of the α, β and τ isoenzymes is thought to contain a Ca^{++} binding region (Parker *et al.*1986), indeed the finding that the activity of the other PKC isoenzymes (δ, ϵ and theca) is Ca^{++} independent (Ono *et al.*1988) and that these isoenzymes do not possess the C2 conserved region (Ono *et al.*1987) suggests that it is this region which contains the Ca^{++} binding site. The finding that some PKC isoenzymes are Ca^{++}-independent may provide an explanation for the ability of phorbol esters to activate PKC in the absence of any detectable elevation in $[Ca^{++}]_i$ (Rink *et al.*1983).

Human and rabbit platelets possess both the α and β subtypes of PKC (of which β is the major subtype) but not the τ subtype (Watanabe *et al.*1988; Pelech *et al.*1991; Tsukuda *et al.*1988). These proteins were resolved using hydroxylapatite column chromatography and found to have a similar chromatographic property to Type II and Type III PKC from the rabbit brain which have been identified as the PKC isoenzymes β and α respectively (the β_1 and β_2 isoenzymes cannot be separated on this technique) (Kikkawa *et al.*1987). Moreover, monoclonal antibodies against the α, β and τ isoenzymes identified only α and β in platelets (Watanabe *et al.*1988).

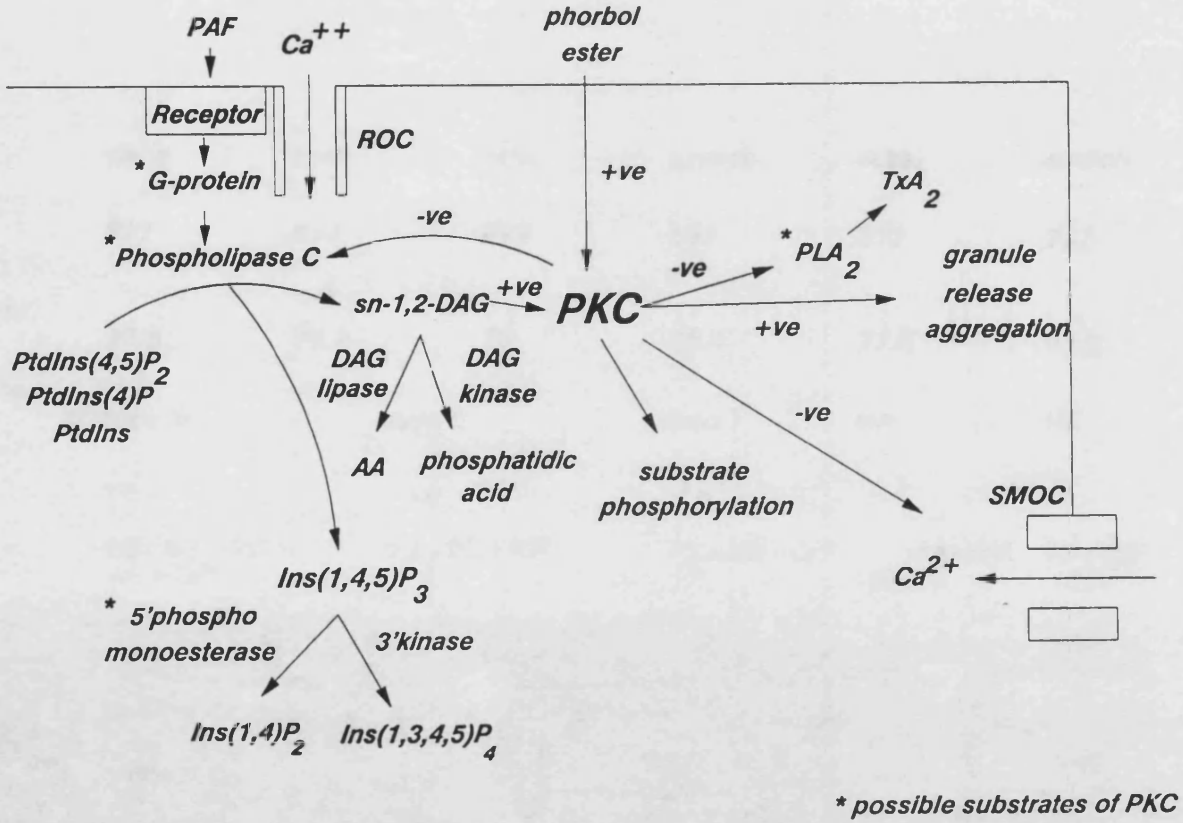


Fig 66 Schematic diagram demonstrating the possible roles of PKC in platelet signal transduction

Subspecies	alpha	$beta_I$	$beta_{II}$	gamma	delta	epsilon	theca
amino-acid residue	672	671	673	697	673	737	592
calculated molecular weight (kDa)	76.8	76.8	78	78.4	77.5	83.5	67.7
chromatographic sub-fraction	type III	type II		type I	NA	NA	NA
chromosome location (human)	17	16		19	?	?	?
activators	PS + DG + Ca^{2+} AA + Ca^{2+}	PS + DG + Ca^{2+}		PS + DG + Ca^{2+} AA	PS + DG + (Ca^{2+})	PS + DG + (Ca^{2+})	PS (DG + Ca^{2+})
location	universal	some cells	many cells	brain + spinal cord	many cells	brain only?	many cells
found in platelets	yes	yes		no	?	?	?

Table 5 Subspecies of PKC from mammalian tissues (Taken in part from Nishizuka 1989).

Platelets have also been found to possess several undefined subspecies of PKC (Tsukuda *et al.*1988) and it is known that at least one of these isoenzymes is insensitive to Ca^{++} (Nishizuka, 1988). Indeed it has recently been suggested that the phosphorylation state of the PKC isoenzyme will determine its Ca^{++} dependence, and in particular phosphorylation of PKC-ß in phorbol ester treated rabbit platelets produces several new forms of the ß-isoenzyme which are Ca^{++}-independent (Pelech *et al.*1991). Whether platelets possess the δ, ϵ or theca-isoenzymes of PKC has not yet been determined.

It has been demonstrated that the PKC isoenzyme ß is less sensitive to Ca^{++} than either α or τ, and that α and τ are less activated by DAG in the presence of PtdS than ß (Nishizuka, 1988; Nishizuka, 1989). Moreover, the τ isoenzyme is readily activated with μM concentrations of AA (without requirement for Ca^{++}, PtdS or DAG) which in comparison has limited ability to activate the PKC ß subtype but will active PKC α in conditions of elevated Ca^{++}. However, it is important to remember that the *in vitro* dependency of PKC on Ca^{++}, DAG and phospholipid varies with the phosphate acceptor (Bazzi and Nelsestuen, 1987), such that comparisons made in limited and defined conditions to compare PKC isoenzymes may not be physiologically accurate.

In this study using low concentration PAF it has been determined that there is a significant elevation of $[Ca^{++}]_i$ in the absence of $Ins(1,4,5)P_3$ or DAG production, these conditions would therefore favour an isoenzyme which was sensitive to Ca^{++} and responsive to any small increases in DAG. At higher concentrations of PAF however, there is a good correlation between DAG elevation and $[Ca^{++}]_i$ elevation and in addition AA is released, providing conditions which would perhaps activate a greater proportion of the isoenzymes. The finding that platelets possess the isoenzyme ß which is thought to be less sensitive to Ca^{++} than either α or τ, and in addition possess another, as yet unidentified, Ca^{++} insensitive isoenzyme, suggests an opportunity for PKC to be activated independently of $Ins(1,4,5)P_3$ or $[Ca^{++}]_i$ elevation. This would introduce the prospect of a novel signal transduction pathway independent of $PtdIns(4,5)P_2$ hydrolysis by PLC, and therefore hydrolysis of any phospholipid which resulted in the generation of DAG would induce PKC activation,

this may include activation of PLD (Loffelholz, 1989). The finding that inhibition of PKC with staurosporine only had an effect when platelets were stimulated with higher concentrations of PAF, would indicate perhaps that only activation of platelets with higher concentrations of PAF was able to create the conditions necessary for PKC activation. The homologous ATP binding site of the PKC isoenzymes would predict that PKC inhibitors which act by competing with ATP for its binding site will inhibit all species of PKC with equal affinity, and indeed this is the case for H-7 (Watanabe *et al.*1988) and for staurosporine (Schaap and Parker, 1990).

4.2.3 To investigate the role of endogenously activated PKC in platelet functional responses using selective inhibitors of PKC.

Although advances were made in the potency of PKC inhibitors with the development of staurosporine (Tamaoki *et al.*1986) and K252a (Yamada *et al.*1988), these inhibitors are still known to be only very poorly selective for PKC, indeed in support of this the IC_{50} values for staurosporine of 8 nM for cAMP-dependent protein kinase, 6 nM for the PTKase $pp60^{v\text{-}src}$ and 3 nM for PKC have been quoted (Tamaoki *et al.*1986; Tamaoki, 1991).

Throughout the course of this study there has been an increasing number of reports describing the development of selective inhibitors of PKC. These selective inhibitors include calphostin C (Kobayashi *et al.*1989) and the Roche compounds Ro 31-8220/002 and Ro 31-7549/001 (Davis *et al.*1989). Calphostin C (UCN-1028C), a compound isolated from *Cladosporium cladosporioides*, was reported to be 1000 time more potent at inhibiting PKC (IC_{50} 50 nM) than other protein kinases including cAMP-dependent protein kinase and tyrosine specific protein kinase (IC_{50} >50 μM) (Kobayashi *et al.*1989). Calphostin C was not able to inhibit PKC activity after PKC had been subjected to proteolysis by calpain (thereby causing removal of the regulatory domain), indicating that calphostin C interacted with the regulatory domain of PKC. The sensitivity of calphostin C to the isozymes of PKC α,ß and τ has been examined and was found to inhibit them all equally (Kobayashi *et al.*1989). Although Ro 31-8220/002 and Ro 31-7549/001 were based on the structure of staurosporine

(Tamaoki *et al.*1986) and K252a (Yamada *et al.*1987) modification of the sugar moieties in the production of the Ro compounds was reported to result in a modulation of potency and selectivity (Davis *et al.*1989). In support of this Ro 31-8220/002 and Ro 31-7549/001 were found to be >80 fold more selective for PKC than cAMP dependent kinase and >1000 fold more selective for PKC than Ca^{++}/calmodulin-dependent kinase. In common with staurosporine both Ro 31-8220/002 and Ro 31-7549/001 compete with ATP for its binding site, it is possible that their modified structure is able to take advantage of the small differences in the otherwise homologous ATP-binding sites of protein kinases to make them selective for PKC. Indeed, it is reported that the C3 region of PKC which contains the ATP binding site has a greater homology between the isoenzymes of PKC, than between PKC and other kinases (Ono *et al.*1987), this would therefore suggest that Ro 31-8220/002 and Ro 31-7549/001 are likely to inhibit the isoenzymes of PKC with equal potency. Using these compounds it is therefore likely that a more accurate insight could be gained into the role of endogenously activated PKC than when using a non-selective inhibitor.

Calphostin C caused a dose-dependent inhibition of 300 nM PAF-induced phosphorylation of the 40-47 kDa substrate of PKC in an intact platelet preparation with an IC_{50} of 1 μM confirming its ability to inhibit PKC activation. Apart from the effects of calphostin C on platelet activation in this study, there has only been one other report regarding the effect of Calphostin C on intact cells (Bruns *et al.*1991). Other studies, have only examined the ability of calphostin C to inhibit phosphorylation of exogenous substrates using an isolated PKC enzyme preparation (Kobayashi *et al.*1989; Tamaoki, 1991). PKC phosphorylation of the EGF receptor has been demonstrated to decrease its affinity for [^{125}I]EGF binding, therefore inhibition of PKC (with Calphostin C) was examined, for its ability to reverse the negative effect of PDBu on EGF binding in A431 cells (Bruns *et al.*1991). In this assay which used intact cells, calphostin C had no effect until around 80 nM after which it increased EGF binding from around 60% (in absence of calphostin c) to around 85% with 250 nM calphostin C, and this reaction was found to be light

dependent. Concentrations of higher than 250 nM calphostin C, inhibited EGF binding and it was stated that this was due to a cytotoxic effect of calphostin C above this concentration and that the cytotoxicity was also light dependent (Bruns *et al.*1991). It was explained by Bruns *et al.* (1991) that similar compounds to calphostin C, namely perylenequinones (in the presence of light) react with molecular oxygen, generate singlet oxygen and form endoperoxides. It is thought that the cytotoxicity of calphostin C may be due to lipid peroxidation by the singlet oxygen. In their study Bruns *et al.* state that there is a 5 fold difference in the concentration of calphostin C which causes PKC inhibition and that which causes cytotoxicity (despite the fact that both reactions are light dependent) and that therefore PKC inhibition is not due to cytotoxicity.

Calphostin C inhibited PKC activation in PAF stimulated platelets, but in contrast to the effects of staurosporine and the selective PKC inhibitors (see below) it also inhibited PAF-induced $[Ca^{++}]_i$ elevation and TxB_2 generation, these experiments were all carried out in light. It is possible that the concentration of calphostin C required to inhibit PKC in an intact cell preparation where it would need to pass through the plasma membrane is higher than required in a isolated enzyme system, as indeed has been demonstrated with platelets in this study. Therefore the narrow margin between the ability of calphostin C to inhibit PKC in the absence of cytotoxic effects and the concentration at which it becomes cytotoxic may have been lost in this study, and its inhibitory effect seen on platelet functional responses may be due to cytotoxicity, although, the fact that there was no leakage of fura-2 from the cells even at the higher concentrations of calphostin C indicate that it did not cause cell lysis. Moreover, in over two years since the first publication on calphostin C (Kobayashi *et al.*1989) only one other non-review paper has been published on this compound (Bruns *et al.*1991). It is possible that due to the cytotoxic side-effects of calphostin C it is not being considered as a good investment for further research.

Both Ro compounds inhibited PAF-induced phosphorylation of the 40-47 kDa major PKC substrate in a dose dependent manner with Ro 31-7549/001 inhibiting phosphorylation with an IC_{50} of 2.5 μM whilst Ro 31-8220/002 inhibited

phosphorylation with an IC_{50} of 0.45 μM. These figures agree with other values for the inhibition of TPA-induced (Davis *et al.*1989) and thrombin-induced (Walker and Watson, 1992) phosphorylation of the 40-47 kDa substrate of PKC in intact platelet preparations. It is known that both PKC and MLCKase phosphorylate MLC (20 kDa) but at distinct sites (Naka *et al.*1983). Indeed it has been demonstrated that, in thrombin stimulated platelets, concentrations of the Ro compounds which inhibit phosphorylation of the 40-47 kDa protein substrate of PKC by 100 % only inhibit MLC phosphorylation by 20%, whereas a concentration of staurosporine which inhibits phosphorylation of the PKC substrate by 100% also inhibits phosphorylation of MLC by 100%. Further, in platelets stimulated by the specific PKC activator TPA (where $[Ca^{++}]_i$ is not elevated and therefore MLCKase not activated), both phosphorylation of the 40-47 kDa substrate of PKC and MLC were totally inhibited by the Ro compounds (Walker and Watson, 1992). This would suggest that the Ro compounds are only inhibiting the PKC-induced phosphorylation of MLC and not the MLCKase induced phosphorylation.

In common with staurosporine the Ro compounds possessed the ability to abrogate the inhibitory effect of activation of PKC with TPA on PAF-induced $[Ca^{++}]_i$ elevation, providing evidence that PKC is the site of action of both these agents. Moreover, and also in agreement with the results for staurosporine, both the Ro compounds were found to have no effect on the maximal height of $[Ca^{++}]_i$ elevation in platelets stimulated with either low or high concentrations of PAF, but to potentiate the duration of the $[Ca^{++}]_i$ signal which was increased by 180% in platelets stimulated with 300 nM PAF whilst only by 50% in platelets stimulated by 3 nM PAF. This finding suggests that inhibition of PKC is removing the negative feedback exerted by endogenously activated PKC upon PAF stimulation therefore potentiating the duration of $[Ca^{++}]_i$ elevation. This finding provides further evidence that PKC is involved in Ca^{++} homeostasis, indeed without the negative feedback exerted by PKC it is possible that Ca^{++} would reach cytotoxic levels.

As predicted, if endogenously activated PKC is exerting a negative feedback over PLC and/or PLA_2, TxB_2 generation was potentiated by inhibition of PKC, again

agreeing with the effects observed with staurosporine. It must be considered however, that *in vivo* the three to five fold increase in TxB_2 production, and thus TxA_2 and prostaglandin endoperoxides, could have considerable effects, depending upon the availability of prostacyclin synthetase or vascular tissue responsive to the vasoconstrictor actions of TxA_2 (Moncada and Higgs, 1986).

In contrast to the above results, but again in agreement with the findings for staurosporine, the Ro compounds caused an inhibition of both dense granule release and aggregation in PAF-stimulated platelets with similar IC_{50}s to those for the inhibition of PAF-induced 40-47 kDa protein phosphorylation in intact platelets. These findings together with the data demonstrating the specificity of these compounds (Davis *et al.* 1989; Walker and Watson, 1992) support the contention that inhibition of dense granule release and aggregation is a result of an inhibition of PKC activity. However, although there is obviously a correlation between inhibition of dense granule release/aggregation and inhibition of the 40-47 kDa protein substrate of PKC, there is no evidence of a cause-effect relationship. Unlike their effects on aggregation and dense granule release, the Ro compounds had no effect on platelet shape change induced by sub-maximal PAF.

Comparing the effects of these selective PKC inhibitors on PAF-induced platelet activation to the effects of the non-selective PKC inhibitor staurosporine, many of the findings were similar. Both selective and non-selective inhibitors potentiated TxB_2 generation and the duration of the Ca^{++} signal whilst inhibiting 5-HT release and platelet aggregation. However, whilst in platelets activated with submaximal concentrations of PAF, staurosporine was found to only partially inhibit platelet aggregation (Watson and Hambleton, 1989), the Ro compounds caused total inhibition of platelet aggregation whilst not affecting platelet shape change. It is possible therefore that staurosporine was inhibiting another protein kinase which was counteracting the effect of PKC inhibition on platelet aggregation. For example, activation of adenylate cyclase increases cAMP levels causing activation of PKA and inhibiting platelet aggregation (Whittle *et al.* 1985), inhibition of PKA by staurosporine would therefore remove this inhibitory effect leading to only a partial possible

inhibition of aggregation by staurosporine. The findings suggest therefore that sub-maximal dose PAF-induced platelet aggregation is PKC dependent whilst PAF-induced platelet shape change is PKC independent.

In conclusion the findings observed using the selective PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002 agree with the previous findings for staurosporine, and support the proposal that PKC has a bifurcating role in activated platelets with endogenously activated PKC exerting a negative feedback on PLC activity, $[Ca^{++}]_i$ elevation and TxB_2 generation whilst exerting a positive role on platelet dense granule release and aggregation.

4.2.4 Determination of the identification and location of annexin (lipocortin/calpactin) isoforms in human platelets

In this study it has been demonstrated that PKC has a role in modulating TxB_2 generation, which is not through modification of the cyclo-oxygenase pathway or TxA_2 synthetase, but which may be due to its regulation of PLA_2 activity. One possibility for the regulation of PLA_2 by PKC, is that activated PKC may phosphorylate an intermediate protein which, depending on its state of phosphorylation, may regulate PLA_2 activity. Candidates for this intermediate protein include members of the annexin family, which are also known by many other terms (Table 6). Annexins have been described as specific inhibitors of PLA_2 (Parente *et al.*1984; Wallner *et al.*1986), although their ability to directly inhibit PLA_2 activity is controversial and may be due to substrate sequestration (Davidson and Dennis, 1989). However, it has been suggested that phosphorylated annexin does not inhibit PLA_2, therefore this may be one way that the action of annexins is regulated by protein kinases (Hirata, 1981).

There is sparse literature on the presence of annexins in platelets. An early paper by Touqui and colleagues identified the major 40-47 kDa protein substrate of PKC as annexin (Touqui *et al.*1986). However, this finding has been disputed for many reasons, firstly, the molecular weights of known annexins (of approximately 35-37 kDa) are different from that of the PKC substrate (40-47 kDa), secondly, in a study

A NOMENCLATURE GUIDE TO THE LIPOCORTIN FAMILY

LIPOCORTIN / ANNEXIN	I	II	III	IV	V	VI	VII	VIII
Copies of consensus sequence	4	4	4	4	4	8	4	4
Phosphorylated by	Protein kinase C EGF receptor insulin receptor	Protein kinase C pp60^{src}				Oncogenes/membrane receptors		
Other names	p35;GIF; Calpactin II; Chromobindin 9	p36;PAP-IV; Calpactin I; Chromobindin 8; Protein-I	PAP-III; 35αCalcimedin Inositol 1,2-cyclic phosphate 2-phosphohydrolase [EC 3.1.4.36]	PAP-II; Chromobindin 4; 32.5K Calelectrin; 35-β Calcimedin; Endonexin I; Protein II; PP4-X	35K Calelectrin; Anchorin CII; IBC; 35γ Calcimedin; VAC-α; PP4; PAP-1; Calphobindin I; Endonexin II	67K Calcimedin; 67K Calelectrin; p68; p70; 73K; Protein III; Chromobindin 20; Calphobindin II	Synexin	VAC-β

Taken from the "Biology of Lipocortin 1" meeting at The Royal Society, November 1991

by Crouch & Lapetina (Crouch and Lapetina, 1986) it was found that both α and τ-thrombin were capable of activating PKC and phosphorylating the 40-47 kDa substrate of PKC, but only α-thrombin was capable of releasing AA (which would not be the case if phosphorylated annexin removed the inhibitory effect over PLA_2), but thirdly and conclusively, the 40-47 kDa substrate has now been cloned and the sequence was found not to be similar to any other known sequence including that of annexin (Tyers *et al.*1988). A recent study however, has demonstrated the presence of annexin V (PAP I) in platelets by the use of ELISA (Flaherty *et al.*1990).

In this study the presence of proteins cross-reacting to antibodies against annexins I, II and V in human platelets has been demonstrated. Immunoblots of U937 cells were probed in parallel since they have previously been demonstrated to contain several annexins, which are increased by protein synthesis when the cells are differentiated in culture (Isacke *et al.*1989). Probing total platelet protein and total U937 cell protein using either antibodies against the whole amino acid sequence of annexin I or against the amino acid sequence 1-188, a protein of 36 kDa was detected in both platelets and U937 cells. However, probing platelets for annexin I using antibodies against the smaller amino acid sequences 24-37 and 38-50, the molecular weight of the proteins detected were around 70 kDa and 55 kDa respectively, therefore different from that detected using the whole amino acid sequence, and despite the fact that annexin I was detected in U937 cells with antibodies against the smaller sequences remained at 36 kDa. It has been suggested that higher molecular weight bands detected by anti-annexin I antibodies may be due to recognition of dimers made up of one native and one proteolysed annexin I molecule (which would account for bands detected of around 60 kDa) or of one annexin I molecule cross-linked to a molecule of another annexin, forming an asymmetrical dimer (which would account for bands of around 66-72 kDa) (Pepinsky *et al.*1989). However, this would not explain why antibodies against the smaller amino acid sequences have detected the higher molecular weight bands but not the 36 kDa band corresponding to the uncomplexed native annexin I. An alternative explanation is that the anti-peptide ABs are recognising other proteins with

sequences similar to that of the 24-37 and 38-50 amino acid sequences in annexin I, although again this would not explain why these antibodies did not detect the protein of 36 kDa which was detected by the anti-annexin I antibody against the whole annexin I amino acid sequence.

The antibody against annexin II detected a protein in platelets which had a larger molecular weight (68 KDa) than that of 35 kDa found in U937 cells which is the usual molecular weight for annexin II. Annexin II is, however, thought to be one member of the annexin family able to form a multimeric protein complex and it may be that it is a dimeric form which is present in platelets (Glenney *et al.*1986). There was no evidence for the presence of annexin IV in platelets, although as there was no positive control for this antibody (ie no annexin IV was found in U937 cells either), the presence of annexin IV in platelets cannot be totally dismissed. However, both platelets and U937 cells were found to possess annexin V at the molecular weight of 32 kDa, in agreement with the findings of Flaherty *et al* (Flaherty *et al.*1990).

The concentration of annexin I was determined in whole platelets lysates by ELISA (Smith *et al.*1990) and found to be around 10 ng/10^9 platelets (6.67 ng/mg protein). The ELISA method has only recently been developed and therefore the number of studies where the concentration of annexin has been assayed is limited (Smith *et al.*1990; Goulding *et al.*1990). In one study untreated human mononuclear leukocytes were found to have a basal level of around 0.8 - 2 ng of annexin I/10^6 cells and human polymorphonuclear leukocytes were found to have a basal level of between 0.7 - 4.2 ng of annexin I/10^6 cells (Goulding *et al.*1990). However, it must be considered that whereas other cell types possess the ability to synthesise annexin I, the annexin in platelets is unlikely to be increased by protein synthesis because of the platelets limited ability to synthesise new protein.

The amount of EGTA/EDTA releasable annexin I from the external surface of the plasma membrane of platelets was found to be less than 0.1 ng/10^9 platelets (<1% of total annexin I) whilst the annexin I in the remaining pellet after removal of the surface annexin was not significantly different to that found in the lysates of untreated platelets. Therefore the findings using the ELISA method correlated well

with the findings for the Western blots. However recently it has been reported that a CHO cell line transfected with the human annexin I gene possessed a novel EDTA resistant form of annexin I, which accounted for the bulk of the surface annexin I (about 5% of total annexin I), and indeed EDTA resistant forms of annexins I,II,IV and VI were reported on the surface of several cultured cell types. It is not possible from the experiments carried out in this study to say whether this EDTA resistant form of annexin I is present on the external surface of the platelet plasma membrane.

Annexins are believed to be released from cells in order to inhibit eicosanoid production and therefore terminate inflammation at an early stage (Flower and Blackwell, 1979; Blackwell *et al.*1982a). However, annexins have also been shown to inhibit blood coagulation *in vitro* by competing with factor Xa and prothrombin for binding to procoagulant phospholipid surfaces (Funakoshi *et al.*1987) and an interesting idea may be that they are functioning as natural anticoagulants. Indeed, an amino acid sequence of annexin, homologous to a sequence from uteroglobulin, has been demonstrated to inhibit platelet aggregation induced by thrombin (Vostal *et al.*1989). The method by which annexins could be released from cells is controversial however, since the amino-acid sequences determined from cDNA clones for at least annexins I and II do not reveal an N-terminal hydrophobic region of a signal peptide usually required for secretion (Wallner *et al.*1986; Pepinsky *et al.*1988). Thus it may be that previous extracellular detection was due to leakage of annexin from dead or damaged cells. More recently however, annexin I has been demonstrated to be secreted by the human prostate gland through what has been described as a novel secretory pathway of high sorting efficiency (Christmas *et al.*1991). The most likely route for the release of annexins from platelets would be if they were contained within platelet granules which are released during platelet activation. In platelets activated using the agonists PAF or thrombin, a Ca^{++} ionophore or phorbol ester which caused varying degrees of platelet granule release, no significant release of annexin I was detected using ELISA (< 1% of total annexin I) and in agreement no release of either annexin I, II or V was detected by Western blotting. However, annexin I was observed to undergo proteolysis, particularly in ionomycin stimulated

platelets, resulting in the appearance of a lower molecular weight band of 32 kDa. In another study stimulation of human platelets with maximal concentrations of ADP, thrombin, collagen and epinephrine did not release annexin V from human platelets and although AA was found to cause some release, it should be cautioned that the concentrations of AA employed in the study of Flaherty *et al* (1-3 mM) would almost certainly have caused some cell lysis which probably accounts for the release (Flaherty *et al.*1990). The finding that activated platelets did not cause any significant release of annexins would suggest that annexins probably do not act as platelet-derived naturally occurring anticoagulants, which indeed would have been contrary to the primary role of platelets in haemostatic plug formation.

It has also been suggested that secretion of the annexins could occur in response to steroids (Goulding *et al.*1990; Blackwell *et al.*1982a). In this study, the glucocorticoid steroid dexamethasone did not cause any detectable release of annexins I,II or V from human platelets, nor did it have any effect on the ability of ionomycin-stimulated platelets to release annexin I.

There is evidence that annexins are involved in signal transduction and may act as second messengers possibly by regulating PLC or PLA_2 by sequestration of their substrates (Machoczek *et al.*1989; Davidson and Dennis, 1989) or they may regulate PKC by interacting in a Ca^{++} dependent manner with phosphatidylserine which is required for PKC activity, however, the physiological significance of their ability to interact with the substrates of PLA_2 and PLC is not known. Recently it has also been suggested that annexin III is inositol 1,2-cyclic phosphate 2-phosphohydrolase (Ross *et al.*1990), again intimating a role for annexins in the regulation of signal transduction pathways. There is now increasing evidence that annexins are cellular substrates for PTKases reviewed in (Russo-Marie, 1991) and PKC (Coméra *et al.*1989; Khanna *et al.*1986). Phosphorylation by these kinases has been found both *in vitro* (Gould *et al.*1986; Coméra *et al.*1989; Khanna *et al.*1986) and in intact cell preparations (Gould *et al.*1986). The fact that signal transduction in platelets has been widely studied and that platelets are a rich source of PKC and the PTKase

$PP60^{c\text{-}src}$ (Golden *et al.*1986) suggest that platelets may be a useful model in which to investigate the role of annexins and their regulation by phosphorylation in signal transduction.

In summary, human platelets have been demonstrated to contain proteins which are recognised by antibodies against annexins I,II and V. These annexins do not appear to be released from the platelets upon stimulation, which agrees with earlier immunological studies which show no evidence for the presence of annexins within secretory organelles of other cell types (Nakata *et al.*1991) and may suggest an intracellular role for annexins. It is not possible to say from this study whether annexins are important in the regulation of PLA_2 activity by PKC in platelets, but having identified their presence, it would be interesting to determine whether they are substrates for PKC or PTKase in an intact platelet preparation.

4.2.5 Characterisation of tyrosine phosphorylation of proteins in PAF-stimulated platelets and investigation into the role of endogenously activated tyrosine kinase(s) in platelet signal transduction using the tyrosine kinase inhibitor genistein.

In this study a Western blotting technique for detecting tyrosine phosphorylated proteins was further developed to improve its sensitivity and was examined and found to be specific for detecting only tyrosine phosphorylated proteins, and not those phosphorylated on either serine or threonine (for example by the action of PKC or PKA).

The temporal relationship of PAF-induced tyrosine phosphorylation was examined over a five minute time course. In unstimulated platelets 4 tyrosine phosphorylated proteins were detected, all of which were in the molecular weight range of 52-62 kDa. This finding agrees with previous studies where resting platelets have been found to possess resident tyrosine phosphorylated proteins within the same molecular weight range (Golden and Brugge, 1989; Nakamura and Yamamura, 1989; Ferrell and Martin, 1988; Nakashima *et al.*1991). Following PAF stimulation, tyrosine phosphorylation of proteins was very rapid with detection of two groups of tyrosine phosphorylated proteins at 5 s after stimulation. One group was between 35-45 kDa

and the other between 66-90 kDa. Within 30 s of stimulation with PAF a further group of tyrosine phosphorylated proteins was detected and these were in the larger molecular weight range of 90-150 kDa. In total, at 30 s following PAF-stimulation around 17 bands of tyrosine phosphorylated proteins could be detected. By determining the relative optical densities of the groups of tyrosine phosphorylated proteins it was possible to examine the patterns of phosphorylation and dephosphorylation over the time course after PAF-stimulation. One of the tyrosine phosphorylated proteins in the group detected in unstimulated platelets was immediately dephosphorylated following PAF-stimulation, although it was then gradually phosphorylated over the ensuing two minutes back to basal levels. This dephosphorylation upon agonist stimulation has previously been found in thrombin stimulated human platelets of a protein of a similar molecular weight, again the immunoreactivity of this protein was quickly restored (Golden and Brugge, 1989). The other three tyrosine phosphorylated proteins in this group were not significantly affected following platelet activation. The two groups of proteins of 35-45 and 66-90 kDa were rapidly phosphorylated, but within two minutes some dephosphorylation of proteins in both groups was detected. Tyrosine phosphorylation of the larger molecular weight group appeared to reach a plateau and did not dephosphorylate over the first two minutes following PAF-stimulation.

Tyrosine phosphorylation of proteins has been detected in platelets stimulated with thrombin (Nakamura and Yamamura, 1989; Ferrell and Martin, 1988; Golden and Brugge, 1989), collagen (Nakamura and Yamamura, 1989; Nakashima *et al.* 1991) and the TxA_2 analogs U46619 and STA_2 (Nakashima *et al.* 1991) indicating that tyrosine phosphorylation is not a specific response to PAF-stimulation. In agreement with the findings for this study, tyrosine phosphorylation of proteins in thrombin-stimulated platelets was also detected in temporal waves, with at least 11 proteins from 27 kDa to 250 kDa phosphorylated following thrombin stimulation (Golden and Brugge, 1989; Ferrell and Martin, 1988; Nakamura and Yamamura, 1989). Indeed, the rate of increase in [^{32}P]phosphotyrosine after thrombin stimulation was 30-100 times greater than in resting cells (Ferrell and Martin, 1988). Therefore it was surprising that in the

study of Dhar & colleagues (Dhar *et al.*1990), that only two tyrosine phosphorylated proteins (of 50 kDa and 60 kDa) could be detected in PAF-stimulated rabbit platelets. They explained the low number of tyrosine phosphorylated bands detected in their study compared to a greater number detected in previous studies (Ferrell and Martin, 1988; Nakamura and Yamamura, 1989; Golden and Brugge, 1989) as being due to the fact that a polyclonal anti-phosphotyrosine antibody had been used in previous studies which might have exhibited broader specificity, compared to the monoclonal anti-phosphotyrosine antibody used in their study. However, this seems an unlikely explanation, as in our study both a monoclonal anti-phosphotyrosine antibody and a polyclonal anti-phosphotyrosine antibody have been employed and were both found to detect at least 17 tyrosine phosphorylated proteins following platelet stimulation with PAF.

The finding that proteins are phosphorylated at different times following PAF-stimulation suggests the activation of more than one PTKase, although the possibility that platelet activation with PAF may be inhibiting more than one tyrosine specific phosphatase (PTPase) should not be dismissed (Smilowitz *et al.*1991; Lerea *et al.*1989). Recently, platelets have been found to contain at least five different PTKases, namely, $pp60^{c\text{-}src}$, $pp60^{fyn}$, $pp61^{hck}$, $pp62^{yes}$ and $pp54/58^{lyn}$ all of which are members of the src family (Huang *et al.*1991; Horak *et al.*1990; Feder and Bishop, 1990). Platelet lysates contain 3-7 fold higher levels of the tyrosine kinase $pp60^{c\text{-}src}$ (which is the cellular homolog of the retroviral transforming protein $pp60^{v\text{-}src}$) than either brain or peripheral blood lymphocytes and 30-50 fold more $pp60^{c\text{-}src}$ than muscle, spleen, thymus, lymph node, bone marrow or erythrocyte lysates (Golden *et al.*1986). Indeed $pp60^{c\text{-}src}$ is believed to account for 0.2-0.4% of total platelet protein levels comparable to structural proteins. $PP60^{fyn}$ is also abundant in platelets, with a 20-40 fold higher concentration than that in fibroblasts, but with a 5-10 fold lower concentration than that of $pp60^{c\text{-}src}$ in platelets (Horak *et al.*1990). Indeed the levels of $pp60^{c\text{-}src}$ are higher than any of the other PTKases so far detected in platelets (Huang *et al.*1991). Due to its relative abundance in platelets, $pp60^{c\text{-}src}$ has now been

purified and enzymatically characterised (Feder and Bishop, 1990), and has been demonstrated to be one of the major proteins phosphorylated *in vitro* in membranes isolated from platelets (Golden *et al.*1986).

The src family of PTKases, unlike those associated with the EGF and PDGF receptors, do not have an extracellular ligand-binding domain, or a membrane spanning domain, which suggests that they function at intracellular sites. $PP60^{c\text{-}src}$ is associated with the cytoplasmic face of cellular membranes (Ferrell *et al.*1990) although PTKase activity has also been detected in the cytosolic fraction (Nakamura *et al.*1985) of platelets. In particular, $pp60^{c\text{-}src}$ has been found to bind to membrane proteins of 32, 50, 92 and 105 kDa in a manner which is dependent on amino-terminal myristylation and which has a strong preference for $pp60^{c\text{-}src}$ amino-terminal sequences, indeed 10^6 of these 50 kDa saturable bindings sites for $pp60^{c\text{-}src}$ have been estimated per platelet (Feder and Bishop, 1991). In platelets $pp60^{c\text{-}src}$ is translocated from the cytosolic to the membrane fraction upon PAF-stimulation (Dhar and Shukla, 1991), although $pp60^{c\text{-}src}$ has also been detected in the membranes of resting platelets (Ferrell *et al.*1990). It is possible that platelet PTKases may be discriminately located on the plasma membrane in order to convey signals from transmembrane receptors which have no intrinsic PTKase activity, for example PAF receptors (Nakamura *et al.*1991) or thrombin receptors (Vu *et al.*1991). Indeed this hypothesis has been demonstrated for the src-like kinase $p56^{lck}$ which is associated with the T lymphocyte CD4 and CD8 surface proteins and which is involved in relaying intracellular signals (Rudd *et al.*1988). Indeed the rapidity with which proteins were tyrosine phosphorylated in PAF-stimulated platelets in this study (<5 seconds) would indicate that PTKase(s) was associated either with the PAF receptor or a closely associated protein.

It must be considered however, that the increase in tyrosine phosphorylation observed in PAF-stimulated platelets could be attributed, even if only in part, to inhibition of phosphotyrosine specific phosphatase (PTPase) activity. It must also be considered that PTPases may function simply to ensure the transient nature of tyrosine phosphorylation. Several protein PTPases have now been identified either

as integral membrane forms or non-receptor forms and sequence analysis has revealed that they possess highly conserved catalytic domains (Tonks and Charbonneau, 1989). The structural organisation of CD45, which is the major leukocyte-specific antigen and a leukocyte specific member of the PTPase family, resembles that of several integral membrane PTKases such as the EGF receptor (Trowbridge *et al.*1991). This would suggest that CD45 and its transmembrane homologs may be capable of initiating a novel mechanism of signal transduction in which the early events involve ligand-induced dephosphorylation of tyrosyl residues in proteins. Whether transmembrane PTPases are receptors, and if so what the identity of their regulatory ligands is, are questions which remain unanswered.

Platelets contain significant amounts of PTPase activity and although the enzymes responsible have not yet been identified over 80% of the PTPase activity is associated with the membrane particulate fraction (Smilowitz *et al.*1991). Subcellular distribution analysis and DEAE Sephacel chromatography suggest that platelets contain more than one form of PTPase (Smilowitz *et al.*1991). PTPase activity in whole platelets can be almost completely inhibited by sodium orthovanadate and ammonium molybdate. In support of this both sodium vanadate and molybdate were found to increase tyrosine phosphorylation of several protein and to stimulate secretion from the dense granules and α-granules of electropermeabilized platelets (Lerea *et al.*1989). In relation to the PAF-induced tyrosine phosphorylated proteins detected in platelets, it is not possible to say whether their phosphorylation is due to PTKase activity or inhibition of PTPase activity. However, the finding that the distinct groups of tyrosine phosphorylated proteins were dephosphorylated at different times following PAF stimulation would support the presence of more than one PTPase enzyme in rabbit platelets. Indeed, one of the tyrosine phosphorylated proteins detected in unstimulated platelets (54 kDa) underwent immediate (detected at 2 s) dephosphorylation following PAF stimulation. In relation to this, $pp60^{c\text{-}src}$ is normally phosphorylated on Tyr-527 *in vivo* which acts as a negative regulator of its kinase activity, and therefore dephosphorylation may be one potential way to activate $pp60^{c\text{-}src}$ and other members of the src family (Okada and Nakagawa, 1989). The molecular

weight of the rapidly dephosphorylated protein in platelets would certainly compare well with members of the src family of PTKases possessed by platelets (Huang *et al.*1991; Horak *et al.*1990; Gutkind *et al.*1990; Feder and Bishop, 1990). Therefore it would be interesting to directly investigate the phosphorylation state and kinase activity of $pp60^{c\text{-}src}$ after platelet activation.

Several inhibitors of PTKases have now been developed, these include the isoflavone compound genistein (Akiyama *et al.*1987), herbimycin A (Uehara and Fukazawa, 1991), erbstatin (Umezaka and Imoto, 1991) and ST638 (Watanabe *et al.*1989). In this study the PTKase inhibitor genistein has been used to investigate the role of endogenously activated PTKases. Firstly, it was established that genistein inhibited PTKase activity and this was achieved by investigating the effect of genistein on tyrosine phosphorylation of proteins following PAF stimulation. Genistein alone was found to have little effect on tyrosine phosphorylation of proteins detected in unstimulated platelets. However, tyrosine phosphorylation of the three groups of proteins which were induced by stimulation with PAF were dose dependently inhibited by genistein. Phosphorylation of the two groups of proteins 35-45 kDa and 66-90 kDa was inhibited by around 90% with 300 μM genistein, whereas this concentration inhibited phosphorylation of proteins of the mw range 90-150 kDa but only by around 50%. This finding further indicates that more than one PTKase is activated following PAF stimulation and that there is differential inhibition of these PTKases by genistein. The IC_{50} value for genistein required to inhibit phosphorylation of the two groups of tyrosine phosphorylated proteins of 35-45 and 66-90 kDa was around 100 μM and that required to inhibit phosphorylation of the 90-150 kDa group was around 300 μM. These IC_{50} values are higher than those quoted for genistein to inhibit autophosphorylation of the EGF receptor in an A431 cell membrane fraction (2.6 μM) or to inhibit phosphorylation of an exogenous substrate in an *in vitro* assay by the EGF receptor (20.4 μM). Moreover, the IC_{50} value for genistein to inhibit the phosphorylation of exogenous substrates in an *in vitro* assay by the $pp60^{src}$ PTKase was 30 μM (Akiyama *et al.*1987). However, other studies investigating the effects of genistein in intact platelets have found concentrations similar to, or higher than,

those used in this study were required to inhibit PTKase activity, (Nakashima *et al.*1991; Gaudette and Holub, 1990; Dhar *et al.*1990), and indeed the IC_{50} value for inhibition of phosphorylation of the EGF receptor *in vivo* was 111 μM (Akiyama *et al.*1987). Genistein is a competitive inhibitor with respect to ATP, and therefore the concentration of ATP in an *in vitro* assay will affect the concentration of genistein required to inhibit PTKase activity (Akiyama *et al.*1987). In the *in vitro* assays described above, 10 μM ATP was present, as opposed to the mM concentrations present in intact cells (Burt *et al.*1976), this would explain the reason why higher concentrations of genistein are required to inhibit PTKase activity in an intact platelet preparation than in the *in vitro* assays.

In comparison to its effects on PTKases, genistein is much less potent at inhibiting other protein kinases. In an *in vitro* assay system, genistein was at least 15 fold more potent at inhibiting PTKase than at inhibiting either PKC or cAMP-dependent kinase (Akiyama *et al.*1987). It is believed that this specificity may be due to the fact that the structure of genistein is unlike ATP, and therefore although competitive with ATP, genistein does not bind to exactly the same site and may in fact bind in multiple places in the reaction pathway. It has been suggested that genistein can discriminate the dissimilarities in the catalytic site for ATP of the different protein kinases (Akiyama *et al.*1987).

Genistein was found to have no effect on basal levels of Ins(1,4,5)P_3, but pre-incubation with genistein inhibited PAF-induced Ins(1,4,5)P_3 elevation measured at 5s after PAF addition with an IC_{50} of 80 μM. Ins(1,4,5)P_3 elevation produced by the hydrolysis of PtdIns(4,5)P_2 upon activation of PLC is one of the earliest events in the signal transduction pathway in PAF stimulated platelets (MacIntyre and Pollock, 1983; Murphy *et al.*1991), indicating that genistein sensitive PTKase(s) is exerting an effect early in the signal transduction pathway. It is unlikely however, that genistein is working by blocking binding of PAF to its receptor as it has been reported that [^{3}H]PAF binding was not affected in the presence of either 200 or 500 μM genistein (Dhar *et al.*1990).

In the presence of EGTA to chelate extracellular Ca^{++}, genistein inhibited PAF-induced $[Ca^{++}]_i$ mobilization from the intracellular stores with an IC_{50} of 120 μM, similar to the IC_{50} value found to inhibit Ins(1,4,5)P_3. From observations made in this thesis it has been suggested that at low concentrations of PAF, there is Ca^{++} influx across the plasma membrane in the absence of Ins(1,4,5)P_3 elevation and therefore that this Ca^{++} influx may be due to a ROC. Genistein was also found to inhibit $[Ca^{++}]_i$ elevation through this pathway although the IC_{50} for this was slightly lower at around 60 μM. Moreover, $[Ca^{++}]_i$ elevation resulting from both mobilization and influx across the plasma membrane, was also inhibited by genistein with an IC_{50} of 100 μM.

The effect of genistein on Ins(1,4,5)P_3 elevation agrees with other studies demonstrating that both genistein (Dhar *et al.*1990) and erbstatin (Salari *et al.*1990) are capable of inhibiting elevation of total [^{3}H]InsP_3 (as opposed to the physiologically relevant Ins(1,4,5)P_3 investigated in this study) in PAF stimulated rabbit platelets. However, this study is the first to demonstrate the effect of genistein on $[Ca^{++}]_i$ elevation in platelets. The inhibitory effect of genistein on Ins(1,4,5)P_3 elevation and $[Ca^{++}]_i$ is in direct contrast to the potentiating effects of the selective PKC inhibitors (Ro 31-7549/001 and Ro 31-8220/002) or the non-selective PKC inhibitor staurosporine on these two signal molecules. This would further suggest that these inhibitors are selective for a particular protein kinase. However, as yet it has not been determined whether genistein is more selective for one particular PTKase than another.

It is interesting to note that whilst genistein has an inhibitory effect on platelet activation induced by several agonists, it appears that in thrombin stimulated platelets PTKase activity may not have such an important role (Nakashima *et al.*1991; Dhar *et al.*1990). Genistein pretreatment has been found to only partially reduce thrombin-induced [^{3}H]InsP_3 elevation (Dhar *et al.*1990), whilst having no effect on thrombin induced sn-1,2-DAG or PA elevation in rabbit platelets (Nakashima *et al.*1991). Moreover, in our laboratory concentrations of genistein found to almost completely inhibit sub-maximal PAF-induced dense granule release had no effect on either

submaximal *α* or *r*-thrombin-induced dense granule release, suggesting that production of these signal molecules and platelet functional responses are, at best, only partially dependent on PTKase activity in thrombin-stimulated platelets.

One study has produced evidence that genistein is responsible for the inhibition of TxA_2 binding to its receptors on platelets (Nakashima *et al.*1991) and therefore agonists, such as collagen, which depend on the production of TxA_2 and on the subsequent binding of TxA_2 to its receptors in order to elicit platelet activation, will not activate platelets in the presence of genistein. In their study Nakashima & colleagues demonstrated that genistein only had a slight inhibitory effect on thrombin-induced tyrosine phosphorylation, signal molecule production and functional responses, and it was suggested that this may be due to the fact that thrombin-induced platelet activation is for the most part independent of TxA_2 production and the subsequent binding to its platelet receptors (Nakashima *et al.*1991). In PAF stimulated platelets, PLC activity is also independent of TxA_2 generation (as has been demonstrated by the lack of effect of indomethacin on PAF-induced sn-1,2-DAG production), however, despite this genistein inhibited tyrosine phosphorylation, signal molecule production and the functional responses: dense granule release, TxB_2 generation and aggregation. Indeed, genistein inhibited TxB_2 generation more potently than other functional responses with an IC_{60} of 10 μM and to such an extent that there is almost complete inhibition of TxB_2 generation by 30 μM genistein. At this concentration of genistein, dense granule release and $[Ca^{++}]_i$ elevation were only marginally inhibited, which would again suggest that PAF-induced platelet activation is independent of TxA_2 production. Moreover it would suggest that neither inhibition of TxA_2 generation nor inhibition of its binding to specific receptors could be responsible for the effects of genistein in PAF-stimulated platelets.

It has previously been demonstrated that as well as being present in surface membrane and cytosol, $pp60^{c\text{-}src}$ is present in the dense granule membranes of platelets (Rendu *et al.*1989) and in the chromaffin granule membrane of adrenal medullary cells (Parsons and Creutz, 1986). This would suggest that PTKase may

have a role in granule secretion, and indeed in this thesis it is demonstrated that inhibition of PTKase activity with genistein inhibits dense granule release.

In agreement with the effect of genistein on PAF-induced functional responses, it has recently been demonstrated that genistein inhibits platelet aggregation in platelets stimulated with other agonists including collagen or the TxA_2 analogs U46619 and STA_2 (Nakashima *et al.*1991) or with PAF (Dhar *et al.*1990) and indeed the PTKase inhibitor erbstatin also inhibits PAF-induced aggregation of rabbit platelets (Salari *et al.*1990). Recent studies suggest a functional relationship between several PTKases and the specific adhesive receptor, namely GPIIb/IIIa, a receptor for fibrinogen and von Willebrand factor (Ferrell and Martin, 1989; Golden *et al.*1990). Agonist-induced platelet activation causes GPIIb/IIIa to undergo a conformational change and allow binding of fibrinogen and von Willebrand factor, the mechanism responsible for this conformational change is unknown, however it is possible that it may be due either directly or indirectly to phosphorylation (Elmore *et al.*1990).

Two separate groups have found that phosphorylation of specific platelet proteins is dependent on GPIIb/IIIa activation and fibrinogen binding (Golden *et al.*1990; Ferrell and Martin, 1989). In one study Golden *et al* found that three proteins of 84,95 & 97 kDa (Golden *et al.*1990) were phosphorylated within 30 seconds which coincided with platelet aggregation (Golden and Brugge, 1989), in another study Ferrel & Martin found that phosphorylation of three proteins of 100, 108 and 126 kDa also coincided with platelet aggregation (Ferrell and Martin, 1989) and it is probable that despite the differences in the electrophoretic mobilities of the proteins the two groups were reporting the same event. Phosphorylation of these proteins could be inhibited if binding of fibrinogen was prevented with the addition of the peptide Arg-Gly-Asp or an inhibitory monoclonal antibody, or indeed if the GPIIb/IIIa receptor was disrupted by EGTA (Golden *et al.*1990). Furthermore, phosphorylation of these proteins was not induced if thrombasthenic platelets congenitally deficient in GPIIb/IIIa were used (Ferrell and Martin, 1989). Moreover, although GPIIb/IIIa activation and fibrinogen binding are necessary for activation of this PTKase activity, they are not sufficient alone, as only in conditions where platelets were stirred and allowed to aggregate

was phosphorylation of these proteins observed (Golden *et al.*1990). It is possible that this aggregation dependent phosphorylation may be required for events associated with aggregation, or downstream of aggregation.

In agreement with these findings, in this study tyrosine phosphorylation of several larger molecular weight proteins of 90-150 kDa was detected and also found to coincide with aggregation in PAF-stimulated platelets. In all experiments investigating tyrosine phosphorylation, platelets were continually stirred and allowed to aggregate, therefore fulfilling the conditions required for phosphorylation of aggregation dependent PTKase substrates (Golden *et al.*1990). Therefore it is possible that the larger molecular weight tyrosine phosphorylated proteins detected in PAF stimulated platelets may be the same as those described in previous studies (Ferrell and Martin, 1989; Golden *et al.*1990). It is possible that genistein, in preventing platelet aggregation, may be subsequently preventing aggregation dependent phosphorylation of these larger molecular weight proteins. Indeed the incomplete inhibition of aggregation by 300 μM genistein, which completely inhibits Ins(1,4,5)P_3 elevation, correlates with the finding that 300 μM genistein only inhibits phosphorylation of these higher molecular weight proteins by 50 percent.

Several studies have investigated the substrates for PTKases activity, and many of the substrates are signal molecules in their own right. Members of the src family contain two protein domains, namely the SH2 and SH3 regions, and it appears that many of the substrates also possess a SH2 sequence and this region may mediate the interaction between the substrate and the PTKase. PLC-γ has been demonstrated to contain a SH2 region and has been shown to be a substrate for both the PDGF and EGF receptor PTKases *in vitro* and *in vivo* (Meisenhelder *et al.*1989). The molecular weight of PLC-γ is around 130 kDa, and indeed the presence of PLC-γ_2 has been detected in platelets (Banno *et al.*1990). Although there is no direct evidence that PLC is a substrate of PTKase in platelets, it is possible that certain receptors may be capable of recruiting a soluble PTKase and activating PLC-γ by a mechanism similar to that described for the EGF receptor. In addition, annexins are also phosphorylated by PTKases both *in vitro* and *in vivo*, and the finding in this study

that platelets possess several members of the annexin family would make them possible contenders as substrates of PTKase. Moreover, it is known that PTKases are capable of autophosphorylation (Feder and Bishop, 1990; Cantley *et al.*1991) and are themselves substrates of other PTKases (Okada and Nakagawa, 1989). The molecular weight of the PTKases known to exist in platelets of 54-62 kDa correlates well with the tyrosine phosphorylated proteins detected in unstimulated platelets of 52-62 kDa in this study. Therefore, considering their abundance in platelets, it is possible that some of the proteins detected by anti-phosphotyrosine antibodies may be PTKases of the src family. Other abundant proteins in platelets are the cytosketetal proteins including actin, myosin, vinculin and tubulin, PTKases are known to associate with the cytoskeleton, and it is possible that these proteins may also be substrates for PTKases in platelets, as found in Rous sarcoma virus transformed cells, reviewed in (Burridge *et al.*1988). It is possible that phosphorylation of these structural proteins may alter the local conformation of the cytoskeleton, possibly increasing anchoring points for enzymes associated with PTKase activity (Grondin *et al.*1991).

Investigations into the interactions between PTKases and PKC have demonstrated that $pp60^{c\text{-}src}$ is a substrate for PKC, being rapidly phosphorylated at Ser-12 in platelets treated with thrombin (Ferrell and Martin, 1988). Although, as phosphorylation at Ser-12 did not alter the kinase activity of $pp60^{c\text{-}src}$ either *in vitro* or *in vivo* the purpose of this phosphorylation is not apparent (Ferrell and Martin, 1988). However, activation of PKC with the phorbol ester TPA did cause an increased translocation of PTKase activity to the platelet membrane (Ishihara *et al.*1989).

Associated with the cytoskeleton of A431 cells are PtdIns kinases, DAG kinase and PLC. Treatment of the cells with EGF increases the activity of these kinases suggesting that their association may play a role in signal transduction induced by EGF by providing a matrix for the various components involved in signal transduction (Payrastre *et al.*1991). Platelet PTKases have also been demonstrated to form multimeric complexes, for example, $pp60^{c\text{-}src}$ and $pp59^{fyn}$ have been demonstrated to

associate with phosphatidylinositol 3-kinase activity within 5 s of platelet activation with thrombin, and this correlates with the increase in 3-phosphorylated phosphoinositides, suggesting a role for nonreceptor PTKases in platelet signal transduction (Gutkind *et al.*1990). PtdIns(3)kinase, only recently described in platelets, is activated upon platelet stimulation with a number of agonists to generate both PtdIns(3,4)P_2 (Nolan and Lapetina, 1990) and PtdIns(3,4,5)P_3 (Kucera and Rittenhouse, 1990). The function of these novel phosphoinositol lipids is unknown at present, although it is thought that they do not provide a substrate for PLC (Nozawa *et al.*1991), but may be signal molecules in their own right (Gutkind *et al.*1990). Moreover, it has been demonstrated that upon platelet activation there is not only a translocation of $pp60^{c\text{-}src}$ (Dhar and Shukla, 1991) to the membrane, but the cytoskeletal activities of PtdIns(4)kinase, PtdIns(3)kinase, DAG kinase and PLCγ are also increased, and it is possible that these enzymes may be regulated by phosphorylation (Gutkind *et al.*1990; Grondin *et al.*1991). Recently the membrane glycoprotein IV (CD36), was found to be physically associated with three platelet PTKases, namely $pp59^{fyn}$, $pp62^{yes}$ and $pp54/58^{lyn}$ (Huang *et al.*1991). The natural ligand of CD36 is uncertain but it is thought possibly to be either thrombospondin or collagen, for review see Shattil and Brugge (1991). Thrombospondin is the major protein secreted from α-granules and is believed to serve as an extracellular bridge between GPIV and the fibrinogen liganded form of GPIIb/IIIa, indeed, the finding that monoclonal antibodies to CD36 induce platelet aggregation and secretion, support a role for CD36 as a signalling receptor (Shattil and Brugge, 1991). This finding therefore supports the possibility that receptors which do not contain intrinsic PTKase activity may, upon stimulation, recruit soluble PTKase(s), which then regulate enzymes involved in signal transduction.

In this study inhibition of PTKase activity with genistein caused an inhibition of Ins(1,4,5)P_3 elevation, which would indicate either an inhibition of PLC activity or substrate availability, and indeed in another study evidence is provided that PTKase regulates the levels of PtdIns(4,5)P_2 and PtdIns(4)P, which are the substrates of PLC (Gaudette and Holub, 1990). The rapidity at which proteins are phosphorylated on

tyrosine, and the finding that Ins(1,4,5)P_3 elevation is inhibited by genistein together with the knowledge that the PAF receptor has no intrinsic PTKase activity would suggest that PTKase is activated at a very early post-receptor level. Indeed, drawing an analogy from the CD36 receptor, it is possible to speculate that activation of the PAF receptor is able to induce and possibly complex with PTKase(s) of the src family, which then regulate critical enzymes of the signal transduction pathway, (figure 67) either by the formation of complexes as has been demonstrated with $pp60^{c\text{-}src}$, $p59^{fyn}$ and PtdIns(3)kinase in platelets (Gutkind *et al.*1990), or by an association of the enzymes to the cytoskeleton at sites proximal to the receptor and PTKase(s) (Grondin *et al.*1991).

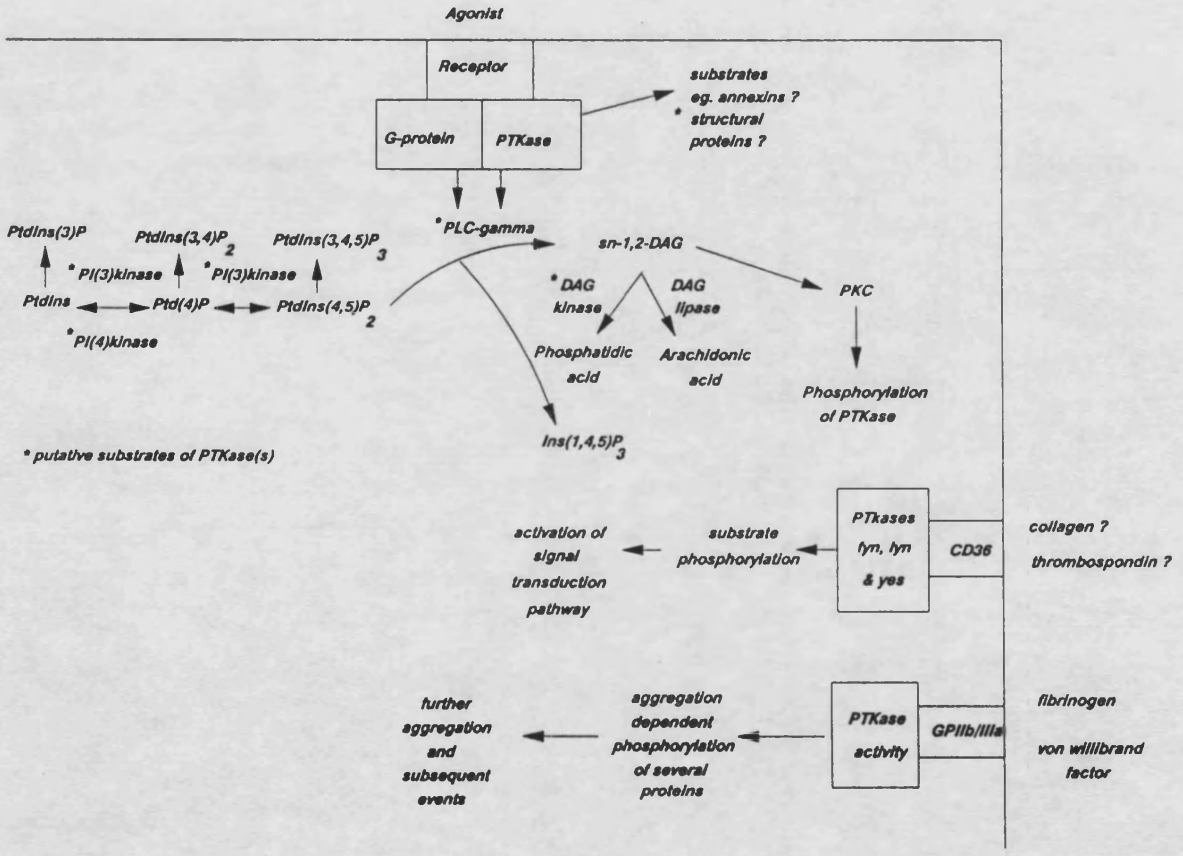


Fig 67 Schematic diagram showing the putative role of PTKase(s) in platelet signal transduction

4.2.6 Summary of Discussion

The aim of this study was to further elucidate the signal transduction pathway of PAF-stimulated platelets and to investigate the role of protein phosphorylation in platelet signal transduction, in particular, to examine the role of PKC and PTKase in PAF-induced platelet activation. This work has been performed by determining the modulatory role of PKC and PTKase on signal molecules elevation, including sn-1,2-DAG, Ins(1,4,5)P_3, $[Ca^{++}]_i$ and protein phosphorylation together with the modulatory role of PKC and PTKase on platelet functional responses including aggregation, dense granule release and Tx generation.

Initially, the role of PKC in modulating platelet functional responses was investigated. To assess where in the signal transduction pathway PKC was exerting its effects platelets were activated either with a receptor-operated stimulus, namely PAF or by direct elevation of $[Ca^{++}]_i$ (ionomycin) or with AA which is converted into TxB_2. PKC activity was modified either by exogenously activating PKC (using either a phorbol ester (TPA) or a membrane permeant DAG) or by inhibiting its action with the use of a non-specific protein kinase inhibitor, namely staurosporine. Activation of PKC caused an inhibition of PAF-induced $[Ca^{++}]_i$ elevation and TxB_2 generation whilst having no significant effect on dense granule release, whilst staurosporine increased the duration of $[Ca^{++}]_i$ and potentiated TxB_2 generation whilst inhibiting dense granule release and platelet aggregation. In ionomycin stimulated platelets modulation of PKC activity had no effect on $[Ca^{++}]_i$ elevation, but in contrast to PAF-stimulated platelets, activation of PKC potentiated both dense granule release and TxB_2 generation whilst staurosporine caused an inhibition of TxB_2 generation and 5-HT release. Thus the effect of PKC modulation in platelets stimulated with ionomycin differs greatly from that in receptor-activated platelets, and makes the use of ionophores together with PKC activators a poor model to use to investigate the role of endogenously activated PKC. Modulation of PKC did not affect AA-induced TxB_2 generation suggesting the site of PKC modulation was not at the level of the cyclo-oxygenase pathway or thromboxane synthetase.

The findings from this part of the study produced evidence that endogenously activated PKC was exerting a negative feedback role in PAF-stimulated platelets, however, when $[Ca^{++}]_i$ elevation was not modified (as in ionomycin stimulated platelets) the relationship between the state of PKC activation and subsequent platelet functional responses corresponded more closely. It appeared that in PAF-stimulated platelets the effect of PKC activation/inhibition on $[Ca^{++}]_i$ elevation was important in modulating TxB_2 generation. Moreover, the findings of this study also demonstrated that a different type of relationship existed between PKC and dense granule release than between PKC and TxB_2 generation in PAF-stimulated platelets.

Next, the temporal and dose-response relationships of PAF-induced changes in the concentration of $[Ca^{++}]_i$, $Ins(1,4,5)P_3$ and sn-1,2-DAG were examined together with the influence of PKC on production of these signal molecules. In high-dose PAF-activated platelets, all three signal molecules increased rapidly and transiently, with the peak $Ins(1,4,5)P_3$ concentration preceding maximal elevation of $[Ca^{++}]_i$ by 5s, supporting the proposal that $Ins(1,4,5)P_3$ is responsible for the mobilization of intracellular Ca^{++} stores. In low-dose PAF-activated platelets there was an increase in $[Ca^{++}]_i$ and dense granule release, without any increase in $Ins(1,4,5)P_3$, sn-1,2-DAG or 40-47 kDa protein phosphorylation. Staurosporine, caused potentiated elevations in the concentrations of $Ins(1,4,5)P_3$, DAG, TxB_2 generation and the duration of the Ca^{++} signal in platelets stimulated with high dose, but not low-dose PAF suggesting that inhibition of PKC was removing the negative-feedback exerted by endogenously activated PKC over PLC. These results suggest that in platelets activated by a low concentration of agonist, mechanisms other than $PtdIns(4,5)P_2$ hydrolysis are utilized for platelet activation, with the possibility that low-dose PAF stimulation may increase $[Ca^{++}]_i$ through the opening of ROCs. In addition, the findings also demonstrate that endogenously activated PKC operates as a powerful negative-feedback regulator of signal molecules.

During the course of this study several selective inhibitors of PKC were developed including calphostin C and the Roche compounds Ro 31-8220/002 and Ro 31-7549/001. Until their development most studies investigating the role of PKC in

intact cells had employed either activators of PKC (such as phorbol esters or membrane permeant DAG) or non-specific inhibitors of PKC. It was important to confirm that the findings made in this study using staurosporine could be attributed to its inhibitory effects on PKC rather than its non-specific inhibitory effects on other protein kinases. Experiments performed with the Ro compounds gave essentially the same results as those using staurosporine. In common with staurosporine the Ro compounds caused a potentiation in both the duration of the Ca^{++} signal and TxB_2 generation, whilst inhibiting both dense granule release and aggregation, confirming a bifurcation role for PKC in PAF-stimulated platelets. One difference however, was in the ability of the Ro compounds to totally inhibit PAF-induced platelet aggregation, whilst staurosporine was found to only be capable of partial inhibition. It is possible that staurosporine was inhibiting another protein kinase which was counteracting the effect of PKC inhibition on platelet aggregation. Experiments performed with calphostin C however produced very different results to those obtained with either staurosporine or either of the Ro compounds. It is interesting that since the time of the first report on calphostin C in 1989, there has been only one other publication regarding calphostin C, where it was found that the margin between the concentration of calphostin C able to inhibit PKC and that which was found to be cytotoxic was very narrow and indeed in an intact cell preparation this margin became even smaller. It is probable therefore that the effects of calphostin C on platelet functional responses observed in this study were not due to its inhibitory effect on PKC.

The finding that PKC was able to modulate PLA_2 activity introduced the possibility that annexin, whose activation has been reported to depend on its state of phosphorylation may be responsible for the regulation of PLA_2 activity. Platelets were demonstrated to possess molecules related to annexins I, II and V, with a total concentration of 10 ng annexin I/10^9 platelets. The annexin-like proteins were intracellular in location, and were not released from granules upon platelet activation with a number of stimulants which possessed separate mechanisms of platelet

activation. Having now identified the presence of annexins in platelets, it would be interesting to determine if they are substrates of either PTKase or PKC in an intact platelet preparation.

Finally, tyrosine phosphorylation of proteins in PAF-stimulated platelets was characterised and the role of endogenously activated PTKase(s) in platelet signal transduction was investigated using the PTKase inhibitor genistein. One group of tyrosine phosphorylated platelets was present in unstimulated platelets, but following PAF stimulation there were several further temporal waves of tyrosine phosphorylation, with two groups of protein substrates phosphorylated within 5s post-PAF and a further group phosphorylated at 30s post-PAF. This finding, together with the observation that genistein inhibited all groups of PAF-induced tyrosine phosphorylation but to different extents, may suggest that PAF is responsible for the activation of more than one PTKase. Genistein inhibited elevation of Ins(1,4,5)P_3 and $[Ca^{++}]_i$ in PAF-stimulated platelets, indicating that endogenously activated genistein-sensitive PTKase(s) has a pivotal role in the early stages of PAF-induced platelet activation. Moreover, genistein also caused the inhibition of PAF-induced TxB_2 generation, dense granule release and aggregation, whether this was due to a direct modulatory role of PTKase(s) on these functional responses, or whether their inhibition was as a result of the inhibition of $[Ca^{++}]_i$ elevation is not yet known. These findings suggest that PTKase(s) holds a modulatory role in the signal transduction pathway, the extent of that role will be revealed with the future identification of which PTKases are activated following agonist stimulation, together with the identification of their substrates and the determination of the endogenous regulators of PTKase activity, coupled with their relationship to platelet PTPase activity.

5. INHIBITORS OF PLATELET FUNCTION AS ANTITHROMBOTIC DRUGS

5.1 Modulation of aspects of platelet activation and signal transduction as mechanisms of regulating platelet function.

Occlusive vascular disease is the major cause of death in the Western World, and there is overwhelming clinical and experimental evidence that platelets contribute to the pathogenesis of stroke, pulmonary embolism, myocardial infarction and atherosclerosis (Harker and Ritchie, 1980; Maseri *et al.*1980). Appreciation of the contribution of platelets in thromboembolic disorders has increased immensely during the past 25 years. With increasing knowledge of platelet activation has been progress into the development of drugs to counteract their pathological role. Platelet functional responses involved in both haemostasis and intravascular thrombosis include a) the adhesion of platelets to the vascular wall at sites of vessel injury, b) the ensuing formation of platelet aggregates, c) the release of pro-aggregatory substances (as well as platelet derived growth factor which will induce proliferation of smooth muscle cells of the vessel wall) from the granules and d) the generation of arachidonic acid metabolites including TxA_2 and PGH_2 (and also possibly PAF). Identification of these components has enabled the development of drugs capable of inhibiting different platelet functional responses. An agent designed as an antithrombotic drug, by its property of inhibiting platelet activation should be selective for platelets over all other cell types. For example, aspirin which is an inhibitor of the cyclo-oxygenase pathway is thought, at certain concentrations, to spare the generation of PGI_2 by endothelial cells whilst inhibiting the TxA_2 generation by platelets, and moreover whilst aspirin irreversibly acetylates platelet cylo-oxygenase, the cells of the vessel wall are able to resynthesize cyclo-oxygenase and therefore regain the ability to generate PGI_2. An ideal antiplatelet drug would, however, be specific for platelets whilst having no affect on the function of any other cell type. Another important consideration in employing an inhibitor of platelet function as an antithrombotic treatment is recognising the balance between controlling thrombosis whilst allowing the role of platelets in haemostasis to proceed in as near normal a manner as possible. As thrombosis is known to be the pathological extension of

normal haemostasis, this may be a difficult criteria to accomplish, unless differences can be distinguished between platelets involved in normal haemostasis and those involved in thrombosis. The balance between the beneficial role of antiplatelet drugs in treating thrombosis and the disadvantage to normal haemostasis will obviously depend on the extent to which platelet function is inhibited. It will also depend on the particular requirements of a patient, for example the benefits of an antiplatelet treatment to a patient who is already suffering from occlusive vascular disease and who is at risk of death from this may outweight the disadvantages of imperfect haemostasis.

In order to assess the ability of a drug to inhibit platelet function, several *in vitro* assays are available. The development of the Born aggregometer (Born, 1962) enabled the ability of putative antiplatelet drugs to be assessed *in vitro* on platelet aggregation. Other *in vitro* assays include adherence of platelets to artificial surfaces, collagen and subendothelium. However, although *in vitro* assays will give some indication of the ability of a compound to inhibit platelet function, *in vivo* testing will allow the effect of agents to be examined on platelets which are present in physiological conditions, with all blood components present, with vessel wall/platelet interactions preserved and in the absence of anticoagulant which is present in most *in vitro* studies. In addition, it allows the drug to be tested at concentrations which can be achieved clinically, the active metabolites of the drug as well as its side effects can also be assessed, together with information on the length of time for which the drug will remain active (Fuster *et al.*1987). The *in vivo* platelet functions are then assessed by measuring bleeding time, platelet survival and turnover and examination of plasma for materials released from platelets such as thromboxane B_2 (Kyrle *et al.*1986).

In addition to identifying the functional responses of activated platelets, which lead to thrombosis, and which can be assayed *in vitro* in the search for antithrombotic drugs, research over the past 20 years has done much to clarify the biochemical mechanisms responsible for stimulus-response coupling. One of the most important breakthroughs was the elucidation of the pathway for AA metabolism, and

the identification of its active metabolites including cyclic endoperoxides and TxA_2 which are responsible for platelet activation and vasoconstriction (Hamberg *et al.*1975). It seems paradoxical therefore that aspirin was identified as a platelet inhibitor (O'Brian, 1968) in advance of it being identified as an irreversible inhibitor of AA metabolism and ironic that in parallel with a further twenty years of platelet research, aspirin has probably been one of the most clinically researched antithrombotic drugs. Other drugs have been developed to modulate the AA pathway and these include inhibitors of the cyclo-oxygenase pathway and TxA_2 synthetase, reviewed in (Fuster *et al.*1987). A highly specific antagonist of the TxA_2/PGH_2 receptors may also prove useful in eliminating the effects of TxA_2 whilst sparing the effects of PGI_2. These drugs however have the disadvantage that they will only affect TxA_2-dependent thrombus formation.

Platelets are now known to be inhibited *in vivo* by the physiological agents PGI_2 and EDRF which are derived from endothelial cells and inhibit platelet activation respectively by increasing cAMP and cGMP levels (Whittle *et al.*1985; Radomski *et al.*1987b). Advantage has been taken of the mechanism of action of these natural inhibitors in the development of longer lasting analogues of PGI_2 working at the same receptor as PGI_2. However, the use of PGI_2 clinically is limited by its host of other biological activities, including a lowering of diastolic pressure by reducing arteriolar resistance due to the potent vascular smooth muscle relaxing properties of prostacyclin (Moncada *et al.*1976). Another drug which works on the same principle of elevating cAMP levels is the phosphodiesterase inhibitor dipyridamole in addition this drug is believed to inhibit vascular and erythrocyte adenosine uptake, elevating the plasma adenosine level, which is also inhibitory to platelets (Packham and Mustard, 1980). Moreover, a nitric oxide (NO) pathway is now believed to be present in platelets, which is inhibited by N^G-Monomethyl-L-arginine, a selective inhibitor of NO synthesis (Radomski *et al.*1990). The NO pathway is activated by several agonists to form NO which modulates platelet activity by increasing cGMP. It is possible that modulation in the activity of this pathway may introduce a novel mechanism of therapeutic significance.

The platelet surface is now known to be the location of the surface-catalysed conversion of pre-enzymes into enzymes in the coagulation cascade, which also includes the dynamic generation of thrombin. Thrombin is thought to be one of the most potent platelet aggregatory agents and its formation on the surface of platelets would anticipate a high local concentration. Therefore research has been invested into thrombin receptor antagonists and drugs which inhibit thrombin formation, most notably heparin which blocks the action of thrombin and through its effect on antithrombin III, inhibits early steps of the coagulation sequence (Packham and Mustard, 1980). However, only thrombin-dependent thrombus formation would be expected to be inhibited by these drugs, and other cell types possessing thrombin receptors would also be affected by thrombin receptor antagonists unless selectivity of a platelet thrombin receptor antagonist could be achieved. The thrombin receptor has however recently been cloned and shown to transmit transmembrane signals by a novel mechanism in which thrombin cleaves its receptor's amino-terminal extension to create a new receptor amino terminus that functions as a bound ligand, this novel mechanism of receptor activation may allow the development of specific antagonists to the thrombin receptor (Vu *et al.*1991). Another receptor which has also recently been cloned is the receptor for platelet-activating factor, which has led to the first direct evidence that receptor activation induces phosphoinositide turnover through G-proteins (Nakamura *et al.*1991). The procurement of human PAF receptor cDNA should create the opportunity for the development of therapeutic drugs.

Agents which inhibit platelet functional responses by other mechanisms include sulfinpyrazone, which is thought to inhibit platelet cyclo-oxygenase in a competitive manner, although it is also believed to have an endothelial protective effect for which no mechanism has been defined. However, whereas sulfinpyrazone has a significant beneficial effect in preventing sudden death due to myocardial infarction in one study it has proven ineffective in two clinical trials where its effect in stroke and unstable angina were monitored. Another drug, ticlopidine however appears more promising, it has been demonstrated to inhibit ADP induced aggregation and also aggregation

and release induced by collagen, epinephrine, AA and thrombin reviewed in (Packham and Mustard, 1980). Although the mechanism of this drug is unknown, it is thought possibly to act on the plasma membrane to alter its reactivity to activating stimuli, perhaps by blocking von Willebrand factor and fibrinogen interactions with platelets, reviewed in (Fuster *et al.*1987).

Ca^{++} is one of the most important signal molecules and is of central importance in cell activation, therefore regulating the elevation of $[Ca^{++}]_i$ could be one mechanism of governing platelet activation. One possibility for an antiplatelet drug could be a Ca^{++} channel blocker, which would impair Ca^{++} movement into platelets. There is now evidence for the presence of receptor operated channels (ROC) in platelets which are capable of carrying a Ca^{++} current and are dependent on ADP (Mahaut-Smith *et al.*1990). The data presented in this study also provide evidence for the presence of a ROC able to carry Ca^{++} ions, coupled to the PAF receptor. Recently, the Ca^{++} channel blocking compound SKF 96365 was demonstrated to inhibit agonist-induced aggregation in thrombin-stimulated platelets (Merritt *et al.*1990), and although this compound was relatively weak, it represents a possible starting point for the development of more potent and selective Ca^{++} channel blockers.

Mobilization of Ca^{++} from intracellular stores has recently been identified as a function of $Ins(1,4,5)P_3$ binding to specific receptors on internal Ca^{++} stores. One of the recent major advances in Ca^{++} signalling has been the cloning and sequencing of the $Ins(1,4,5)P_3$ receptor and the identification of the homology between the $Ins(1,4,5)P_3$ and the ryanodine receptor, which is the Ca^{++} releasing channel of skeletal muscle (Supattapone *et al.*1988; Mignery *et al.*1989; Furuichi *et al.*1989). Platelet activation by most agonists is believed to cause Ca^{++} mobilization in advance of Ca^{++} influx (Alonso *et al.*1991), suggesting that influx is dependent on some aspect associated with Ca^{++} mobilization, for example the production of second messengers, or the state of filling of the intracellular dischargeable Ca^{++} stores (Sage *et al.*1989). Therefore controlling Ca^{++} mobilization may also subsequently regulate the Ca^{++} influx component. Having identified the $Ins(1,4,5)P_3$ receptor it may now be possible to develop receptor antagonists to prevent Ca^{++} mobilization, indeed the

receptor is known to bind heparin which is able to displace Ins(1,4,5)P_3 from the receptor and inhibit Ca^{++} mobilization. The development of synthetic, non metabolizable, stable analogues of Ins(1,4,5)P_3 may also provide a starting point for the production of an Ins(1,4,5)P_3 receptor antagonist, however, these antagonists would need to be cell permeant. The finding that there is a variation in the binding affinity of Ins(1,4,5)P_3 for its receptors on different tissues and that the IC_{50} value for heparin to displace Ins(1,4,5)P_3 from its binding sites is different between rat cerebellum and bovine adrenal cortex suggests that there may be heterogeneity between Ins(1,4,5)P_3 receptors (Willcocks and Nahorski, 1989). Distinct binding sites for Ins(1,4,5)P_3 for different tissues would raise the possibility of developing an Ins(1,4,5)P_3 receptor antagonist specific for the Ins(1,4,5)P_3 receptor of platelets, which has recently been characterised (O'Rourke and Feinstein, 1990). An alternative mechanism of controlling Ins(1,4,5)P_3 induced Ca^{++} mobilization is to control the removal of Ins(1,4,5)P_3. Ins(1,4,5)P_3 is metabolised by inositol 5'phosphomonoesterase to the inactive metabolite Ins(1,4,)P_2, therefore potentiating the activity of this enzyme would reduce Ins(1,4,5)P_3 levels and limit $[Ca^{++}]_i$ elevation. A recent finding of interest is that the Ins(1,4,5)P_3 receptor is a substrate for cAMP-dependent kinase, suggesting this may be one way in which cAMP levels are able to regulate $[Ca^{++}]_i$ levels (Supattapone *et al.* 1988).

It is becoming increasingly obvious that platelets as well as being involved in haemostasis and the pathological extension of this, namely thrombosis together with the conditions which result from thrombosis, also participate in and exacerbate many other pathological conditions (section 1.3). It is possible that the production of an antiplatelet drug would prove beneficial in the alleviation of these conditions, particularly in relation to the number of potential cell stimulants including platelet derived growth factor, ADP and serotonin which are released from platelet granules following agonist stimulation.

5.1.1 Regulation of protein kinase C

The results of this thesis and earlier studies demonstrate that increasing $[Ca^{++}]_i$ with a divalent cation ionophore and activating PKC with a phorbol ester causes a synergistic increase in platelet activation. This system was thought to mimic the subsequent events of PtdIns(4,5)P_2 hydrolysis (ie $[Ca^{++}]_i$ elevation by Ins(1,4,5)P_3 and PKC activation by DAG) in agonist stimulated platelets, and suggests that the development of a specific, potent PKC inhibitor, may be useful as an antithrombotic drug. However, unfortunately this may not be the case, as in this thesis it has been demonstrated that the use of an ionophore together with a phorbol ester represents a poor model for agonist-induced platelet activation.

In this and in other studies, it has been demonstrated that exogenous activation of PKC in agonist-stimulated platelets causes an inhibition of the two signal molecules Ins(1,4,5)P_3 and sn-1,2-DAG providing evidence that PKC exerts a negative feedback role. Therefore it would be expected that inhibition of endogenously activated PKC would inhibit this negative feedback, and there would be an increase in the signal molecules Ins(1,4,5)P_3 and sn-1,2-DAG, as indeed has been observed in this study using both the non-selective inhibitor staurosporine and the selective PKC inhibitors Ro 31-7549/001 and Ro-8220/002, further confirming that PKC exerts a negative feedback either directly or indirectly over PLC. Inhibition of PKC in agonist stimulated platelets also results in a potentiation of the duration of $[Ca^{++}]_i$ elevation and TxB_2 generation, but an inhibition of dense granule release and aggregation. This would suggest that PKC has a bifurcating role in platelet activation induced by a physiological agent, exerting a positive influence over dense granule release and aggregation but a negative influence over Ca^{++} homeostasis and thromboxane generation. Therefore, *in vivo* an inhibitor of PKC rather than having an antithrombotic effect would induce a substantial increase in TxA_2 and prostaglandin endoperoxides, which could have considerable effects, both in the recruitment of platelets to a potential thrombotic region and also due to the ensuing vasoconstrictor properties of TxA_2.

It is possible however, with the realisation that PKC is a family of isoenzymes, that distinct PKC isoenzymes may be responsible for regulating different platelet functional responses. For example, if a PKC isoenzyme was recognized which could regulate dense granule release (particularly if this enzyme was unique to platelets) and an inhibitor was identified which could selectively inhibit this isoenzyme, this inhibitor in association with a cyclo-oxygenase pathway inhibitor, such as aspirin, could be potentially useful as an antithrombotic treatment.

5.1.2 Regulation of tyrosine phosphorylation

Although the presence of PTKases in platelets has been known for some time (Tuy *et al.*1983) it was only relatively recently recognised that PTKase was activated following platelet stimulation with agonists such as thrombin (Ferrell and Martin, 1988), collagen (Nakamura and Yamamura, 1989) and PAF (Dhar *et al.*1990; Murphy and Westwick, 1992). The role of PTKase activity, is unknown in platelets, although the rapidity with which substrates are phosphorylated following agonist stimulation, together with the effects of PTKase inhibitors suggests that it may be involved in signal transduction.

In support of a role for PTKase activity in signal transduction is the finding that several inhibitors of PTKase are able to inhibit both platelet signal molecule elevation and platelet functional responses in agonist stimulated platelets suggesting that endogenously activated PTKase(s) has a positive role in signal transduction (Dhar *et al.*1990; Salari *et al.*1990). Indeed, the results presented in this thesis would suggest that PTKase(s) has a regulatory role at an early stage of the signal transduction pathway in PAF-stimulated platelets, as inhibition of PTKase activity caused an inhibition in the elevation on Ins(1,4,5)P_3. The identification of PTKase(s) present in platelets however, together with their characterisation both in terms of their substrates and how they are regulated is at a very early stage. Indeed in other cell types PLCγ is known to be a substrate of PTKase, and therefore may be regulated by phosphorylation. It is not known if PLCγ is a substrate of PTKase in

platelets, but if it is the case, this may introduce a mechanism of controlling platelet activation at an early stage of the signal transduction pathway.

The finding that members of the src family associate with membrane receptors and that they have a key role in platelet signal transduction, coupled with the possibility that platelets (as has been demonstrated in T-lymphocytes) may possess a PTKase which is specific to platelets, offers an original therapeutic approach in the design of novel anti-thrombotic compounds. Moreover, the fact that PTKase(s) appear to regulate an early stage of the signal transduction pathway suggests that a PTKase inhibitor would suppress all facets of platelet function.

In association with PTKases, is the growing awareness that PTPases, rather than merely being responsible for the transient nature of tyrosine phosphorylation following cell activation, may have a more important role. The possibility of receptors with intrinsic PTPase activity or which are complexed with cytosolic PTPases introduces the prospect of a novel signal transduction pathway, initiated by dephosphorylation of tyrosine phosphorylated proteins. Very little is known about the PTPases which are possessed by platelets (Smilowitz *et al.*1991), except that their inhibition leads to an increase in granule release (Lerea *et al.*1989). It is possible therefore that PTPases may also play an important regulatory role in platelet signal transduction, both independently and in association with PTKases, such that the regulation of tyrosine phosphorylation may also provide an additional mechanism of controlling platelet functional responses and therefore assist in the design an antithrombotic treatment.

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Murphy, C.T., Elmore, M., Kellie, S., and Westwick, J. (1991) The relationship between cytosolic calcium, sn-1,2-diacylglycerol and inositol 1,4,5-trisphosphate elevation in PAF stimulated rabbit platelets. Biochem. J., *278*:255-261.

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ABSTRACTS

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The relationship between cytosolic Ca^{2+}, *sn*-1,2-diacylglycerol and inositol 1,4,5-trisphosphate elevation in platelet-activating-factor-stimulated rabbit platelets

Influence of protein kinase C on production of signal molecules

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The temporal and dose–response relationships of platelet-activating-factor (PAF)-induced changes in the concentrations of cytosolic Ca^{2+} ($[Ca^{2+}]_i$), Ins(1,4,5)P_3 and 1,2-diacylglycerol (DAG) were examined. In addition, phosphorylation of protein kinase C (PKC) substrate (40–47 kDa protein) was determined. In high-dose PAF-activated platelets, all three signal molecules increased rapidly and transiently, with the peak Ins(1,4,5)P_3 concentration preceding maximal elevation of $[Ca^{2+}]_i$ by 5 s. In low-dose PAF-activated platelets there were large increases in $[Ca^{2+}]_i$ and dense-granule release, without any increase in Ins(1,4,5)P_3 and DAG or 40–47 kDa protein phosphorylation. Staurosporine, a non-specific PKC inhibitor, produced enhanced elevations in the concentrations of Ins(1,4,5)P_3, DAG and thromboxane B_2, and the duration of the Ca^{2+} signal in platelets stimulated with a high dose, but not a low dose, of PAF. These results suggest there are both phospholipase C-dependent and -independent changes in Ca^{2+} homoeostasis. Endogenously activated PKC regulates the formation of signal molecules.

INTRODUCTION

Receptor-activated hydrolysis of PtdIns(4,5)P_2 via the action of phospholipase C generates Ins(1,4,5)P_3 (Berridge & Irvine, 1984) and *sn*-1,2-diacylglycerol (DAG) (Rittenhouse-Simmons, 1979). Both of these products are thought to be intracellular signal molecules which regulate certain aspects of cell activation (Berridge, 1984, 1987).

Ca^{2+} mobilization from intracellular stores is induced in permeabilized platelets by Ins(1,4,5)P_3 (O'Rourke *et al.*, 1985; Brass & Joseph, 1985), whereas influx of Ca^{2+} across the plasma membrane via signal-molecule-operated Ca^{2+} channels may be regulated by Ins(1,4,5)P_3 working in concert with one of its phosphorylated products, Ins(1,3,4,5)P_4 (Irvine, 1989, 1990). The only well-characterized source of Ins(1,4,5)P_3 is the hydrolysis of PtdIns(4,5)P_2, whereas its removal is regulated either by phosphorylation to Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3 kinase or by degradation to Ins(1,4)P_2 by 5-phosphomonoesterase (Batty *et al.*, 1985; Connolly *et al.*, 1987; Mitchell *et al.*, 1989).

The hydrolysis of (poly)phosphoinositides is probably the major source of DAG (Rittenhouse-Simmons, 1979), although an additional source may be via the action of phospholipase D on phosphatidylcholine (Pai *et al.*, 1988; Loffelholz, 1989). In platelets, however, the presence and role of phospholipase D have yet to be verified (Rubin, 1988). Receptor-activated DAG elevation is transient, probably owing to its phosphorylation by DAG kinase to phosphatidic acid (Tysnes *et al.*, 1988) and its degradation by DAG lipase to fatty acid and glycerol (Bell *et al.*, 1979; Mahaevappa & Holub, 1986). This short-lived increase in DAG is thought to activate protein kinase C (PKC) (Nishizuka, 1984; Lapetina *et al.*, 1985), a family of ubiquitously distributed isoenzymes which have been implicated as having many different roles, including the regulation of cell activation (Nishizuka, 1984, 1988). The main substrate for PKC in platelets is a 40–47 kDa protein of unknown function (Kaibuchi *et al.*, 1983; Touqui *et al.*, 1986).

Little work has been carried out on intact platelet preparations to study the relationship between the signal molecules cytosolic Ca^{2+}, Ins(1,4,5)P_3 and DAG. In this study we have determined the temporal and dose–response relationships of platelet-activating-factor (PAF)-induced elevations in $[Ca^{2+}]_i$ to changes in the levels of Ins(1,4,5)P_3 and DAG in intact rabbit platelets. As an alternative experimental approach we have used the potent, although non-specific, PKC inhibitor staurosporine (Tamaoki *et al.*, 1986) to examine the role of endogenously activated PKC in the regulation of receptor-stimulated changes in the concentrations of $[Ca^{2+}]_i$, Ins(1,4,5)P_3 and DAG.

MATERIALS AND METHODS

Materials

Prostacyclin was generously provided by Dr. B. J. R. Whittle, Wellcome Laboratories, Beckenham, Kent, U.K. It was stored solid and dry at −20 °C, and stock portions were dissolved in 0.5 M-Tris/HCl (pH 10.5) and were stored at a concentration of 40 μg/40 μl at −20 °C. Immediately before use a portion was thawed and diluted as required in 0.01 M-Tris/HCl (pH 10.5), and then stored on ice. DAG assay reagents system (code RPN 200), [^{3}H]Ins(1,4,5)P_3 assay system (code TRK 1000), [^{32}P]ATP (3 Ci/mmol), 5-hydroxy[^{14}C]tryptamine (5-HT) creatinine sulphate (54 mCi/mmol, 50 μCi/ml) and [^{32}P]P_i (5 mCi/ml) were purchased from Amersham International, thin-layer plates (silica gel 60 HPTLC aluminium; 20 cm × 20 cm) were purchased from Merck, and X-ray film was from Fuji. Fura-2 AM (acetoxymethyl ester; cell-permeant) obtained from Molecular Probes, Eugene, OR, U.S.A. was stored dry at

Abbreviations used: DAG, 1,2-diacylglycerol; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concn.; $[Ca^{2+}]_e$, extracellular Ca^{2+} concn.; PAF, platelet-activating factor; PKC, protein kinase C; HBT, Hepes-buffered Tyrode's solution; TxB_2, thromboxane B_2; 5-HT, 5-hydroxytryptamine; PMA, phorbol 12-myristate 13-acetate.

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−20 °C. A stock solution at a concentration of 5 mM in anhydrous dimethyl sulphoxide was also stored at −20 °C. PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was supplied by Bachem (Bubendorf, Switzerland). A stock solution of 10 mM made up in chloroform/methanol (4:1, v/v) was stored at −20 °C, and working solutions (0.1 mM) were prepared in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to which 2.5 mg of BSA/ml had been added. WEB 2086, a gift from Dr. H. Huer (Boehringer, Ingelheim, Germany), was dissolved in phosphate-buffered saline. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma, dissolved in dry acetone and stored at −20 °C. Staurosporine was purchased from Fluka Chemicals, Glossop, U.K., dissolved in dimethyl sulphoxide and stored at −20 °C. Thromboxane B_2 (TxB_2) standard was obtained from Sigma, [^{3}H]TxB_2 (125 Ci/mmol, 50 nCi/ml) was from New England Nuclear, and anti-(rabbit TxB_2) antibody was generously supplied by Dr. F. Carey, ICI, Macclesfield, Cheshire, U.K.

Preparation of platelets

Washed rabbit platelets were prepared as described for human platelets by Poll & Westwick (1986), by a modification of an original method by Blackwell *et al.* (1982). The resulting platelet pellet from this preparation was suspended in Hepes-buffered Tyrode's (HBT) solution (10 mM-Hepes, 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 0.5 mM-Na_2HPO_4, 5.5 mM-glucose and 0.25% BSA, pH 7.4) and the platelet count was adjusted.

Protein phosphorylation

Platelets (8×10^9/ml) were incubated for 2 h with 200 μCi of [^{32}P]P_i/ml of platelets, washed in HBT, and the platelet count was adjusted to 10^9/ml. Batches of platelets (300 μl; 10^9/ml) were equilibrated at 37 °C in the presence of 1 mM-$[Ca^{2+}]_e$. Platelets were incubated with a dose range of PAF, and activation was terminated with 300 μl of 2 × Laemmli (1970) SDS reducing gel-sample buffer (100 °C) at 30 s after PAF addition. The samples were then boiled for 5 min, and centrifuged at 8000 *g* for 2 min to remove any particles. Proteins (100 μg/track) were separated by SDS/10%-polyacrylamide-gel electrophoresis; protein bands and molecular markers were stained with Coomassie Blue, and [^{32}P]phosphorylated bands were observed by autoradiography. The phosphorylated bands were excised and quantified by liquid-scintillation counting.

Measurement of DAG production

Platelet DAG production was determined with the DAG assay reagents system (Amersham International, RPN 200), which used an adaptation of the method described by Preiss *et al.* (1986). Briefly, 1 ml portions of platelets (10^9/ml) were dispensed into cuvettes and the $[Ca^{2+}]_e$ was adjusted to 1 mM. Platelets were continually stirred and allowed to equilibrate to 37 °C before addition of drug/vehicle control. All drugs were incubated for 2 min before addition of PAF. DAG production was stopped at pre-determined times by removing 0.8 ml into 3 ml of ice-cold methanol/chloroform (2:1, v/v), and samples were extracted by a modification of the method of Bligh & Dyer (1959). The assay employed was a radio-enzymic assay using *Escherichia coli* DAG kinase and defined mixed-micelle conditions to solubilize the DAG present and allow its quantitative conversion into [^{32}P]phosphatidic acid in the presence of [^{32}P]ATP. The sensitivity of the assay was < 10 pmol, and the working range was 31–1000 pmol/tube.

Determination of Ins(1,4,5)P_3 production

Experiments were carried out as described for DAG determination. The reaction was terminated and Ins(1,4,5)P_3 was extracted by mixing the cell suspension with 0.2 vol of ice-cold 20% (v/v) $HClO_4$ and after 20 min sedimenting the proteins by centrifugation. The supernatant was then titrated to pH 7.5 with 1.5 M-KOH containing 60 mM-Hepes buffer and Universal indicator. The Ins(1,4,5)P_3 present in the samples was determined by use of a binding assay which employed unlabelled Ins(1,4,5)P_3, [^{3}H]Ins(1,4,5)P_3 and a bovine adrenal-membrane preparation (Challiss *et al.*, 1988). The sensitivity of the assay was < 0.2 pmol, and the working range of Ins(1,4,5)P_3 detected by this assay was 0.2–25 pmol/tube.

Determination of $[Ca^{2+}]_i$

Platelets were loaded with 2.5 μM-fura-2 AM for 30 min at 37 °C, the platelet suspension was then washed in HBT to remove excess fura-2 AM, and the platelet count adjusted to 2×10^8/ml. To measure the changes in $[Ca^{2+}]_i$, fluorescence readings were recorded at 37 °C with an Aminco Bowman spectrophotofluorimeter (excitation at 339 nm; emission at 500 nm; 4 nm slit width) fitted with a thermostatically regulated cuvette compartment holder with stirring attachment. Samples (1 ml) of platelets were dispensed into cuvettes, and $[Ca^{2+}]_e$ was adjusted to 1 mM. The Ca^{2+}–fura-2 fluorescence was calibrated as we have previously described by using EGTA quench (Ward & Westwick, 1988). Platelets were equilibrated to 37 °C before the addition of drugs or drug vehicles, which were incubated for 2 min before addition of PAF.

Measurement of percentage [^{14}C]5-HT release

Platelets (8×10^8/ml) were incubated with 200 nCi of [^{14}C]5-HT/ml, then washed in HBT, and the count was adjusted to 2×10^8 platelets/ml. $[Ca^{2+}]_e$ was adjusted to 1 mM, and platelets were equilibrated to 37 °C before addition of a drug or vehicle control. To determine 5-HT release from the platelets, sub-samples of platelets (400 μl) were withdrawn 3 min after addition of the agonist into ice-cooled tubes containing 40 μl of 100 mM-EDTA and 30 μM-indomethacin. The samples were centrifuged at 12000 *g* for 2 min and 2 × 100 μl samples of the supernatant were removed and the amount of [^{14}C]5-HT was determined by liquid-scintillation spectroscopy. Percentage 5-HT release was determined as previously described (Holmsen *et al.*, 1981).

Measurement of TxB_2 generation

TxB_2 generation was determined in 1 ml samples of platelets (2×10^8/ml), with $[Ca^{2+}]_e$ adjusted to 1 mM. Platelets were equilibrated to 37 °C before addition of drug or drug vehicle, which was added 2 min before PAF. To terminate TxB_2 generation, sub-samples of platelets (400 μl) were withdrawn at pre-determined times (3 min unless otherwise stated) into 40 μl of ice-cold 100 mM-EDTA/30 μM-indomethacin. The samples were centrifuged, the supernatant was removed and the TxB_2 content determined by radioimmunoassay (Johnston *et al.*, 1984).

Statistics

Data in the Figures are means ± S.E.M. of at least three experiments each performed in either duplicate or triplicate. To determine the levels of significance between sets of data, a paired Student's *t* test was employed. The levels of significance are denoted by $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. The Bonferroni correction was applied where necessary (Wallenstein *et al.*, 1980).

RESULTS

PAF-induced elevation of $[Ca^{2+}]_i$ and concentrations of Ins(1,4,5)P_3 and DAG

Addition of 300 nM-PAF induced a rapid elevation of platelet $[Ca^{2+}]_i$ from a basal concentration of 152 ± 11 nM ($n = 30$) to a

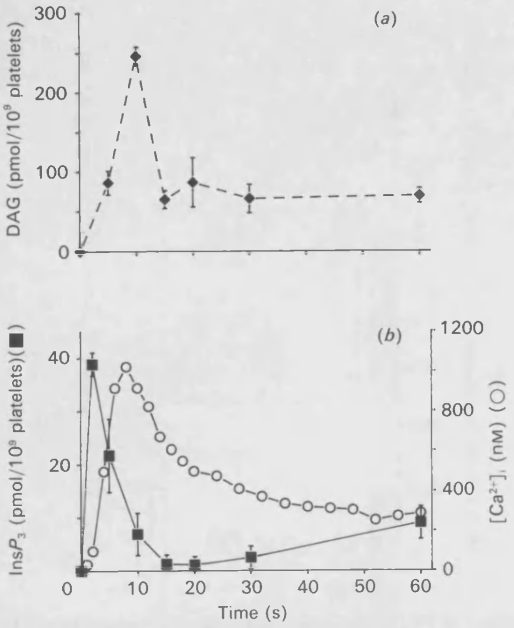

Fig. 1. Time courses for the production of DAG, Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ in 300 nM-PAF-stimulated platelets

DAG production induced by 300 nM-PAF is shown in (*a*), and $[Ca^{2+}]_i$ elevation (○) together with Ins(1,4,5)P_3 elevation (■) are shown in (*b*). The values in all Figures were obtained by subtracting the basal pre-PAF value from those obtained at the stated times after administration of 300 nM-PAF. The values are means ± S.E.M. of at least three experiments. The S.E.M. bars are absent when symbols are larger than the S.E.M.

Table 1. Effect of 2 min pre-treatment with the PAF receptor antagonist WEB 2086 on PAF-induced Ins(1,4,5)P_3 and DAG generation

	Generation (% of control)	
Treatment	Ins(1,4,5)P_3*	DAG‡
Vehicle + 300 nM-PAF	100	100
30 nM-WEB 2086 + 300 nM-PAF	24.0 ± 14	22.5 ± 16
300 nM-WEB 2086 + 300 nM-PAF	4.7 ± 3.2	0

* Measured 5 s after PAF addition.
‡ Measured 10 s after PAF addition.

peak elevation of 1023 ± 72 nM at 7.3 ± 0.6 s (n = 13) after PAF addition. This elevation was transient, and quickly declined (Fig. 1). Similarly, in the same platelet preparations 300 nM-PAF induced a rapid elevation in the concentration of Ins(1,4,5)P_3, with a peak increase at 2 s of 38.9 ± 2.2 pmol/10^9 platelets above a basal level of 9.77 ± 1.72 pmol/10^9 platelets (Fig. 1). This elevation rapidly declined to basal levels by 15 s after PAF, followed by a gradual increase at 30 and 60 s after PAF.

Superimposition of the Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ time curves demonstrates that the Ins(1,4,5)P_3 peak clearly precedes the peak $[Ca^{2+}]_i$ by approx. 5 s.

Platelets had a basal concentration of 117 ± 3 pmol of DAG/10^9 platelets. After addition of 300 nM-PAF, there was a rapid and transient increase in the platelet DAG concentration, to a peak at 10 s with a value of 280 ± 52 pmol/10^9 platelets (n = 9) above basal. After 10 s the concentration of DAG rapidly declined, and by 15 s reached a level above basal, at which it remained for the duration of the time course (60 s) (Fig. 1).

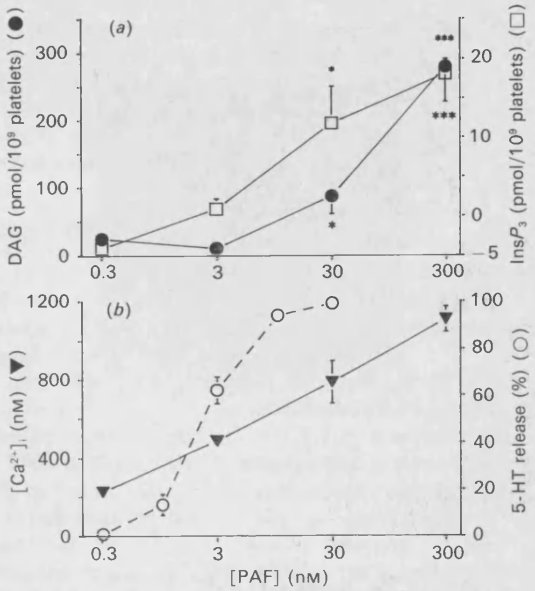

Fig. 2. Effect of increasing concentrations of PAF on production of DAG and Ins(1,4,5)P_3 (*a*) and $[Ca^{2+}]_i$ elevation and 5-HT release (*b*)

DAG incubations were terminated at 10 s after PAF, and those for Ins(1,4,5)P_3 at 5 s. 5-HT release was terminated at 3 min after activation and $[Ca^{2+}]_i$ is the peak elevation after stimulation. The values were obtained by subtracting the basal pre-PAF value from those obtained after addition of PAF. The values are means ± S.E.M. of at least three experiments.

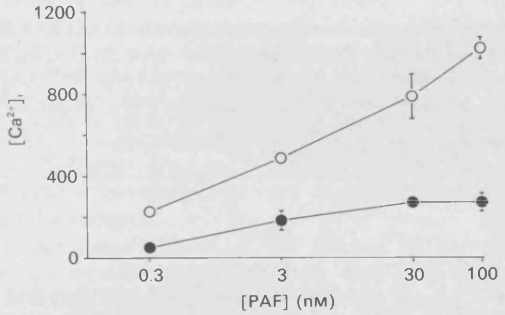

Fig. 3. Effect of replacing 1 mM-Ca_e^{2+} with 2 mM-EGTA on PAF-induced $[Ca^{2+}]_i$ elevation

$[Ca^{2+}]_i$ is the value obtained by subtracting the basal pre-PAF value from the peak post-PAF value. Each value represents the mean ± S.E.M. of at least three experiments each performed in triplicate. Key: ○, control; ●, +2 mM-EGTA.

Addition of PAF vehicle did not produce changes in the basal concentrations of $[Ca^{2+}]_i$, Ins(1,4,5)P_3 or DAG (results not shown).

Table 1 demonstrates that pre-treatment of the platelets with the PAF-receptor antagonist WEB 2086 (300 nM) inhibited to the same degree PAF-induced elevation of Ins(1,4,5)P_3 and DAG.

Dose–response relationship of PAF-induced platelet activation

The relationship of the concentrations of the three putative signal molecules, $[Ca^{2+}]_i$, Ins(1,4,5)P_3 and DAG, to the release of 5-HT was determined (Fig. 2).

PAF is a powerful dense-granule releasing agent, and the dose–response relationship is shown in Fig. 2(*b*). The higher

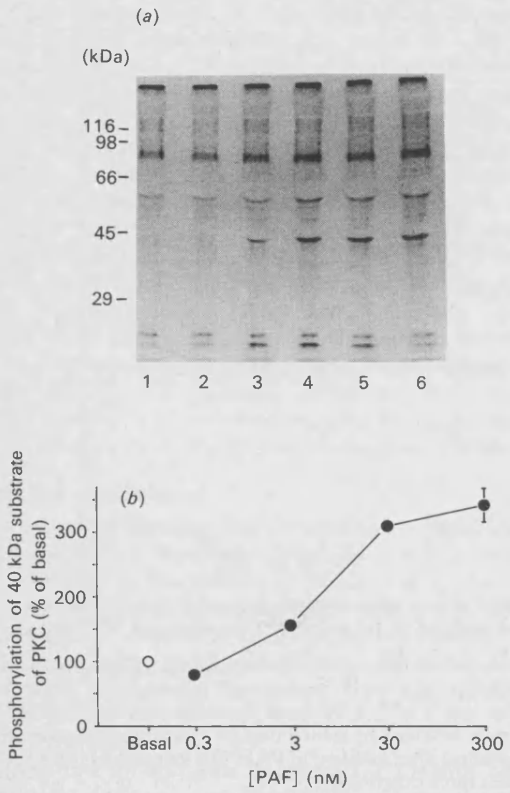

Fig. 4. Effect of increasing concentrations of PAF on phosphorylation of the 40 kDa substrate of PKC

(*a*) Autoradiograph showing phosphorylation of the 40 kDa substrate of PKC by PAF vehicle (track 1) or by 0.3, 3, 30 and 300 nM-PAF respectively (tracks 2–5) and with 100 nM-PMA (track 6). The phosphorylated PKC substrate band was excised and subjected to scintillation counting, and the results are given in (*b*) as a percentage of phosphorylation with PAF vehicle.

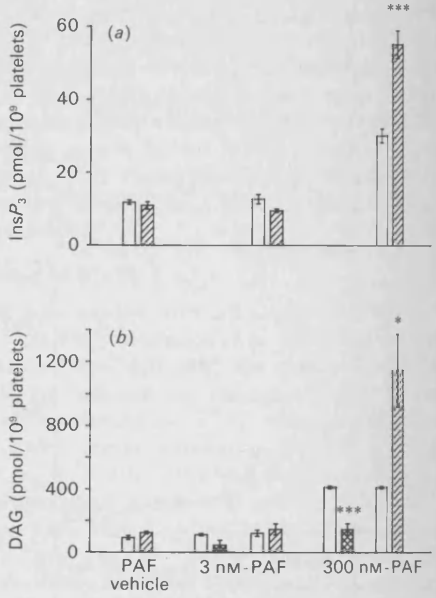

Fig. 5. Effect of PKC activation or inhibition on generation of DAG and Ins(1,4,5)P_3 induced by 3 nM- and 300 nM-PAF

PKC was pre-activated with 100 nM-PMA or pre-inhibited with 1 μM-staurosporine. DAG incubations (*b*) were terminated 10 s after the addition of PAF, and Ins(1,4,5)P_3 incubations (*a*) were terminated at 5 s. Each value represents the mean ± S.E.M. of at least three experiments each performed in duplicate. Key: □, control; ▨, 1 μM-staurosporine; ⊠, 100 nM-PMA.

doses of PAF, 30 and 300 nM, which are maximal and supramaximal for 5-HT release, produced highly significant ($P < 0.005$) elevations in $[Ca^{2+}]_i$, in addition to a rise in the concentrations of Ins(1,4,5)P_3 (measured at 5 s) and DAG (measured at 10 s) (Fig. 2*a*). However, a lower concentration of PAF, such as 3 nM, which induced approx. 60% 5-HT release and a 400 nM increase above the basal $[Ca^{2+}]_i$, induced no detectable increase in either Ins(1,4,5)P_3 or DAG. Replacing $[Ca^{2+}]_i$ with the Ca^{2+} chelator EGTA inhibited $[Ca^{2+}]_i$ elevation at all concentrations of PAF tested (Fig. 3). At the higher concentrations of PAF, 30 and 300 nM, and to a lesser extent at 3 nM, $[Ca^{2+}]_i$ elevation was due to two components, the major one being dependent on $[Ca^{2+}]_e$, whereas the minor one was independent of $[Ca^{2+}]_e$. At the lower concentration of 0.3 nM-PAF, however, $[Ca^{2+}]_i$ elevation was completely dependent on the presence of $[Ca^{2+}]_e$. In relation to the findings for DAG, activation of PKC, measured as phosphorylation of its 40 kDa substrate, was increased dose-dependently with increasing concentrations of PAF; 3 nM-PAF induced some activation of PKC after 30 s, but, again in agreement with the findings for DAG, no phosphorylation with 0.3 nM-PAF was detected (Fig. 4).

These results suggest that there is a good correlation between both PtdInsP_2 hydrolysis/Ca^{2+} mobilization and granule release at higher doses, but not at lower concentrations of PAF.

Relationship between PKC and PAF-induced platelet activation

To explore the role of endogenous activators of PKC on the regulation of PAF-induced formation of Ins(1,4,5)P_3, DAG, TxB_2 and changes in Ca^{2+} homoeostasis, the effect of the potent PKC inhibitor staurosporine on these parameters was examined: 1 μM-staurosporine was used, as we have previously demonstrated that this concentration inhibits at least 70% of PKC activation (Murphy & Westwick, 1990).

Ins(1,4,5)P_3 and DAG. Pretreatment of the platelets with 1 μM-staurosporine did not modify the basal concentrations of either Ins(1,4,5)P_3 or DAG (Fig. 5). Thus PKC does not exert a tonic control over these parameters in the unstimulated platelets. The effect of staurosporine on PAF-stimulated formation of Ins(1,4,5)P_3 and DAG was dependent on the concentration of PAF employed. Staurosporine produced a 2- and 3-fold enhancement of Ins(1,4,5)P_3 and DAG formation respectively in response to 300 nM-PAF (Fig. 5). When 3 nM-PAF was used, which alone failed to produce an increase in Ins(1,4,5)P_3 or DAG over the basal levels, pretreatment with staurosporine did not produce any detectable increase over this.

Pretreatment of the platelets with the PKC activator PMA (100 nM) produced a significant decrease in basal and PAF-stimulated levels of DAG (Fig. 5).

Ca^{2+} homoeostasis. Pretreatment of platelets with 1 μM-staurosporine did not modify the basal $[Ca^{2+}]_i$ of platelets (results not shown). In addition, preincubation of platelets with staurosporine had no significant effect on the rate of elevation or the maximum elevation of $[Ca^{2+}]_i$, stimulated with either 3 nM- or 300 nM-PAF (Fig. 6). However, the duration of the Ca^{2+} signal, measured as the time taken to return to half-maximum elevation above basal ($t_{\frac{1}{2}}$), was significantly potentiated by staurosporine for both concentrations of PAF (Fig. 6). The increase in $t_{\frac{1}{2}}$ in platelets stimulated with 3 nM-PAF was only 30%, in contrast

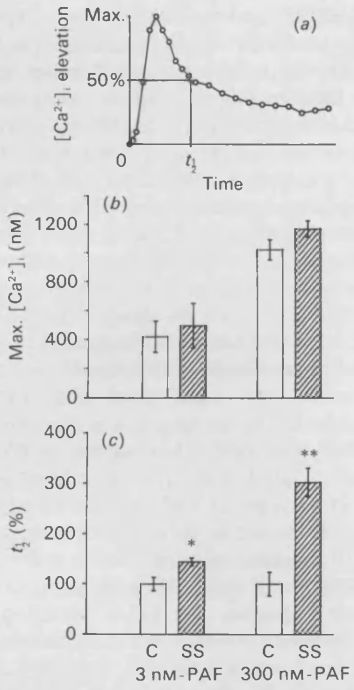

Fig. 6. Effect of staurosporine on PAF-induced $[Ca^{2+}]_i$ elevation

Effect of 1 μM-staurosporine on the peak elevation of $[Ca^{2+}]_i$ or on the $t_{\frac{1}{2}}$ (time for decay of elevated $[Ca^{2+}]_i$ to half-maximal elevation over basal) in platelets stimulated with 3 nM- or 300 nM-PAF. (*a*) Representative trace of fluorescence output transformed to $[Ca^{2+}]_i$ from fura-2-loaded platelets, demonstrating the concepts of peak $[Ca^{2+}]_i$ and $t_{\frac{1}{2}}$. (*b*) Effect of staurosporine on maximal $[Ca^{2+}]_i$ elevation. (*c*) Effect of staurosporine on the $t_{\frac{1}{2}}$. $[Ca^{2+}]_i$ is the value obtained by subtracting the basal pre-PAF value from the peak post-PAF value. Each value represents the mean ± S.E.M. of at least three experiments each performed in triplicate. Key: □, control (C); ▨, staurosporine (SS).

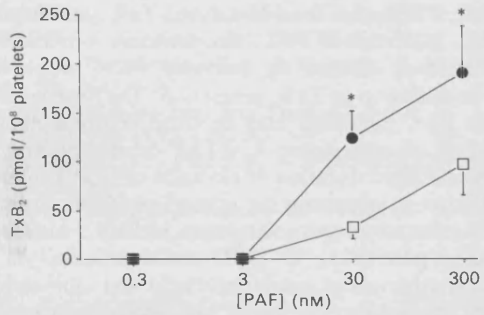

Fig. 7. Effect of 1 μM-staurosporine on PAF-induced TxB_2 generation

TxB_2 generation was determined 3 min after addition of PAF. The points represent means ± S.E.M. of three experiments each performed in triplicate. Key: □, control; ●, staurosporine.

with the 200% increase in $t_{\frac{1}{2}}$ observed in 300 nM-PAF-stimulated platelets.

TxB_2 formation. Only trace amounts of TxB_2 (< 1 pmol/ml) were detected after platelet activation with either 0.3 nM- or 3 nM-PAF, and these levels were not affected by staurosporine (Fig. 7). However, at higher concentrations of PAF (30–300 nM) there was a dose-dependent increase in the TxB_2 generated, and this was potentiated at least 2-fold with staurosporine (Fig. 7).

To determine whether the staurosporine-enhanced TxA_2 formation was responsible for the increased DAG formation (Fig. 5), the effect of indomethacin on this system was examined. Pretreatment of the platelets with 2.8 μM-indomethacin, which caused > 99% inhibition of TxB_2 formation, had no significant ($P > 0.05$) effect on basal, PAF-induced or staurosporine-enhanced levels of DAG (results not shown).

DISCUSSION

We have examined the relationship between the three putative signal molecules, namely Ins(1,4,5)P_3, DAG and $[Ca^{2+}]_i$, in PAF-stimulated rabbit platelets, in both the absence and the presence of an inhibitor of PKC.

A very rapid and transient elevation of all three signal molecules was induced by the addition of a high concentration of PAF. The concentration of Ins(1,4,5)P_3 reached a peak at 2 s after PAF addition, which preceded the peak concentration of $[Ca^{2+}]_i$ by 5 s. In addition, the peak concentration of Ins(1,4,5)P_3 was 45 pmol/10^9 platelets, which is equivalent to 4.5 μM (assuming that 10^9 platelets have a cytosolic volume of 1 μl; Rittenhouse & Sasson, 1985), close to the 5 μM-Ins(1,4,5)P_3 that is required for maximum release of $^{45}Ca^{2+}$ from permeabilized human platelets (Brass & Joseph, 1985) and membrane vesicles (O'Rourke *et al.*, 1985). Thus our observations in intact platelets, using high concentrations of PAF, provide good support for the proposal that Ins(1,4,5)P_3 is responsible for mobilizing intracellular Ca^{2+} (Berridge & Irvine, 1984).

The time course and the amount of DAG formed, compared with Ins(1,4,5)P_3, suggests that PtdInsP_2 hydrolysis may not be the only source of DAG. Possible additional sources include the hydrolysis of other phosphoinositides or the action of phospholipase D on phosphatidylcholine (Loffelholz, 1989). Whatever the source of DAG, the 3-fold increase over basal reported in the present study with PAF-stimulated rabbit platelets corresponds well to the 2–3-fold DAG elevation found in thrombin-stimulated human platelets, when the same assay for DAG was used (Preiss *et al.*, 1986). The parallel inhibition of PAF-induced elevations of Ins(1,4,5)P_3 and DAG by WEB 2086 (Casals-Stenzel *et al.*, 1986) indicates that these events are closely coupled to a single class of PAF receptor.

The higher concentrations of PAF (30 and 300 nM) produced dose-related increases in the amounts of $[Ca^{2+}]_i$, Ins(1,4,5)P_3, DAG, 40 kDa phosphorylation and granule release. However, sub-maximal concentrations of PAF (0.3 and 3 nM), which caused considerable dense-granule release and elevation of $[Ca^{2+}]_i$, failed to induce a detectable increase over basal in the concentration of either Ins(1,4,5)P_3 or DAG. Thus, in platelets activated with sub-maximal levels of PAF, neither the Ins(1,4,5)P_3-dependent mobilization of Ca^{2+} from intracellular stores nor the DAG-dependent activation of PKC would be expected. The lack of $[Ca^{2+}]_i$ elevation in the absence of $[Ca^{2+}]_e$ at low levels of PAF, together with the decreased phosphorylation of PKC substrate with sub-maximal levels of PAF, support this.

These findings have several implications. Firstly, at higher concentrations of PAF, there is a good correlation between products of PtdInsP_2 hydrolysis and platelet activation, but at the lower concentrations of PAF, platelet activation, including Ca^{2+} mobilization, occurs independently of PtdInsP_2 hydrolysis. Indeed, in other cell types a lack of correlation between PKC activation and functional response, or Ins(1,4,5)P_3 elevation and $[Ca^{2+}]_i$ elevation, has been found. A lack of correlation between DAG accumulation and superoxide-anion production after stimulation of neutrophils with either concanavalin A (Rider & Niedel, 1987) or *N*-formylmethionyl-leucyl-phenylalanine (Koenderman *et al.*, 1989) has been observed. In vascular smooth-muscle cells stimulated with PAF, the time to peak

$[Ca^{2+}]_i$ elevation is reached before any elevation in Ins(1,4,5)P_3 (Schwertschlag & Whorton, 1988), and it has been suggested that other mechanisms are responsible for regulating PAF-induced Ca^{2+} mobilization, namely a receptor-operated Ca^{2+} channel independent of phosphatidylinositol polyphosphate hydrolysis. Similarly, in ADP-stimulated platelets Ca^{2+} entry was found clearly to precede internal discharge, and it was suggested that receptor occupancy is closely coupled to the opening of plasma-membrane bivalent-cation channels (Sage *et al.*, 1989). Furthermore, in hepatocytes the formation of Ins(1,4,5)P_3 has been found to require a higher concentration of receptor-operated hormone than does $[Ca^{2+}]_i$ elevation (Lynch *et al.*, 1989). This finding has been explained as either Ins(1,4,5)P_3 elevation and $[Ca^{2+}]_i$ elevation not being causally related, or by the presence of spare receptors for agonists that mobilize Ca^{2+}. Moreover, PAF has been shown to increase $[Ca^{2+}]_i$ in neutrophils where phospholipase C activation, and therefore Ins(1,4,5)P_3 production, have been inhibited by pertussis toxin (Naccache *et al.*, 1985).

To determine the role of PKC in the regulation of early post-receptor events, we used the potent, although non-specific, PKC inhibitor staurosporine (Tamaoki *et al.*, 1986; Watson *et al.*, 1987). A concentration of 1 μM-staurosporine was selected, as we had previously shown that this concentration inhibited, by at least 70%, 300 nM-PAF-induced PKC-substrate phosphorylation (Murphy & Westwick, 1990). Both DAG and Ins(1,4,5)P_3 production induced by 300 nM-PAF were significantly potentiated by PKC inhibition; however, there was no effect on Ins(1,4,5)P_3 or DAG levels in platelets stimulated with 3 nM-PAF. Previous studies, using phorbol esters or membrane-permeant DAG to activate PKC before agonist or fluoride stimulation in platelets, showed that some functional responses were inhibited by pre-activation of PKC (Poll & Westwick, 1986; Poll *et al.*, 1986; Krishnamurthi *et al.*, 1986). This led to the theory that, when activated, PKC caused a negative feedback, inhibiting generation of both DAG and Ins(1,4,5)P_3 at the level of production (at either phospholipase C or a controlling G-protein) (Watson & Lapetina, 1985; Zavoico *et al.*, 1985). The level of Ins(1,4,5)P_3 was further suppressed as activation of PKC increased its metabolism to Ins(1,4)P_2 (Molina y Vedia & Lapetina, 1986; Connolly *et al.*, 1986).

We suggest that in our system, where PKC is activated by higher concentrations of PAF, DAG and Ins(1,4,5)P_3 levels are depressed by negative feedback. However, when PKC is inhibited the negative feedback is removed and a full elevation of DAG and Ins(1,4,5)P_3 induced by 300 nM-PAF stimulation results. At lower concentrations of PAF no negative feedback would be exerted by PKC, and therefore the inhibition of PKC would, as observed, have no effect. However, although the findings suggest that PKC is exerting its effect on the production of Ins(1,4,5)P_3 and DAG, as they are both formed from the hydrolysis of PtdInsP_2 by phospholipase C, the findings do not dismiss the possibility that PKC may also have a role in the removal of Ins(1,4,5)P_3 and DAG (King & Rittenhouse, 1989). Another interesting point from these findings is that the activator of PKC, namely DAG, appears to be self-regulating.

We also attempted to correlate the effect of PKC inhibition on generation of DAG or Ins(1,4,5)P_3 with the effect on the functional responses of $[Ca^{2+}]_i$ elevation and TxB_2 generation. Firstly, staurosporine had no significant effect on either 3 nM- or 300 nM-PAF-induced peak $[Ca^{2+}]_i$ elevation, despite an increase in Ins(1,4,5)P_3 on stimulation by 300 nM-PAF. This could be due to the limits of detection of $[Ca^{2+}]_i$ elevation being reached after stimulation with 300 nM-PAF, and therefore any further increase in $[Ca^{2+}]_i$ being indistinguishable, or that maximal intracellular Ca^{2+} mobilization had already been achieved. There was no increase in Ins(1,4,5)P_3 at 3 nM-PAF, however, and although it would have been possible to detect an increase in $[Ca^{2+}]_i$ at this concentration of PAF, no significant increase was observed. However, when investigating the $t_{\frac{1}{2}}$ of the decay of $[Ca^{2+}]_i$ it was found that PKC inhibition produced a 200% increase in the $t_{\frac{1}{2}}$ of elevated $[Ca^{2+}]_i$ in 300 nM-PAF-stimulated platelets, but only a 30% increase in $t_{\frac{1}{2}}$ in platelets stimulated with 3 nM-PAF. These findings are in agreement with the work of Watson & Hambleton (1989), where an increase in $t_{\frac{1}{2}}$ of $[Ca^{2+}]_i$ decay was observed in thrombin-stimulated platelets pretreated with 1 μM-staurosporine, despite no evidence of a potentiation of Ins(1,4,5)P_3 (Watson *et al.*, 1987).

These results taken together also suggest that PKC may be acting to restore the elevated $[Ca^{2+}]_i$ to basal levels, as was shown by Poll & Westwick (1986) and Pollock *et al.* (1987) in human platelets. Alternatively, our findings are in agreement with those of King & Rittenhouse (1989), in respect of PKC inhibition potentiating Ins(1,4,5)P_3 levels, although unlike us they found that this caused an increase in $[Ca^{2+}]_i$ elevation. Moreover, King & Rittenhouse (1989) found no potentiation in thrombin-induced DAG levels by 10 μM-staurosporine. This is interesting, because Bishop *et al.* (1989) found that, with a high concentration of the PKC inhibitor sphingosine, the DAG elevation induced by thrombin was abolished, but at lower concentrations of sphingosine the DAG level was potentiated, and they attributed the inhibition by sphingosine to an inhibition of proximal portions of the signal-transduction pathway at this higher concentration of inhibitor. Therefore it may be that the concentration of 10 μM-staurosporine used in the study by King & Rittenhouse (1989), which is 10-fold higher than that used in our study, has masked any potentiatory response which may have been observed at a lower concentration of staurosporine.

We also examined the effect of staurosporine on PAF-induced TxB_2 generation. At lower concentrations of PAF (0.3–3 nM), only trace amounts of TxB_2 were detected, but at higher PAF concentrations (30 nM and 300 nM) TxB_2 was dose-dependently increased, and this increase was potentiated by inhibition of PKC. Earlier studies have shown that pre-activation of PKC causes an inhibition of agonist-induced TxB_2 generation (Murphy & Westwick, 1989) and fluoride-induced TxB_2 generation (Poll *et al.*, 1986). Inhibition of PKC, and therefore inhibition of the negative feedback exerted by activated PKC, would indeed predict a potentiation in TxB_2 generation. The increase in TxB_2 induced by PKC inhibition may be attributed to a number of events. Firstly, phospholipase A_2 is Ca^{2+}-dependent, and it may be that the sustained duration of elevation of $[Ca^{2+}]_i$ induced by PKC inhibition is increasing the activation of this enzyme and thus causing the observed potentiation in TxB_2. Alternatively, the increased production of DAG could provide, via DAG lipase, increased levels of arachidonic acid and ultimately TxB_2 production (conversely, the use of indomethacin provided evidence that the increase in TxA_2 was not responsible for the increase in DAG production). Finally, it is also possible that PKC is exerting a direct effect on phospholipase A_2 or a closely coupled GTP-binding protein (Silk *et al.*, 1990) or that it is affecting TxB_2 production by modulating a family of proteins called lipocortins which, if present, may be responsible for inhibiting phospholipase A_2 activity (Touqui *et al.*, 1986).

Several conclusions can be drawn from the present work. The relationship between the concentrations of the signal molecules and the platelet functional responses is very dependent on the dose of PAF employed. In high-dose-PAF-activated platelets we have shown that the elevation of Ins(1,4,5)P_3 clearly precedes the $[Ca^{2+}]_i$ elevation, supporting the proposal that Ins(1,4,5)P_3 is responsible for this mobilization of intracellular Ca^{2+} stores. In contrast, in low-dose-activated platelets, elevation of $[Ca^{2+}]_i$ and

granule release occurs in the absence of detectable increases in the concentration of Ins(1,4,5)P_3 or DAG, as well as an absence of Ca^{2+} mobilization from intracellular stores or PKC-substrate phosphorylation.

These results suggest that in platelet activation induced by low concentrations of agonist, mechanisms other than polyphosphoinositide bisphosphate hydrolysis are utilized for platelet activation. The existence of these separate mechanisms is supported by experiments with an inhibitor of PKC, which results in a marked potentiation of DAG, Ins(1,4,5)P_3 and TxB_2 production as well as a protracted Ca^{2+} signal induced to a much greater extent by the higher doses of PAF. Finally, the results demonstrate that endogenously activated PKC operates as a powerful negative-feedback regulator of signal molecules.

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Comparison of the role of protein kinase C in platelet functional responses induced by three different mechanisms, PAF, ionomycin and arachidonic acid

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The role of protein kinase C (PKC) in modulating platelet activation has been examined in platelets pre-incubated with either the PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or the non-specific protein kinase inhibitor, staurosporine. In order to determine where in the signal transduction pathway PKC is exerting its effect platelets were activated either with a receptor-operated stimulus platelet activating factor (PAF) or by direct elevation of $[Ca^{2+}]_i$ (ionomycin) or with arachidonic acid which is converted into thromboxane B_2 (TxB_2). In PAF-stimulated platelets activation of PKC inhibited both $[Ca^{2+}]_i$ elevation and TxB_2 generation but had no effect on 5-hydroxytryptamine (5-HT) release whilst staurosporine increased the duration of $[Ca^{2+}]_i$ elevation and potentiated TxB_2 generation but inhibited 5-HT release. In ionomycin-stimulated platelets modulation of PKC had no effect on $[Ca^{2+}]_i$ elevation but in contrast to PAF-stimulated platelets PKC activation caused potentiation of TxB_2 generation and 5-HT release whilst inhibition of PKC caused inhibition of TxB_2 generation and 5-HT release. Modulation of PKC did not affect arachidonic acid-induced TxB_2 generation. These findings suggest that in receptor activated platelets endogenously activated PKC is exerting a negative feedback role, however, when $[Ca^{2+}]_i$ elevation is not modified by PKC activation or inhibition (such as in ionomycin stimulated platelets) the relationship between the state of PKC activation and subsequent platelet functional responses corresponds more closely. The findings from this study suggest a different relationship between PKC and TxB_2 generation than between PKC and dense granule release in PAF-stimulated platelets.

Introduction

Receptor-mediated activation of platelets initiates a series of biochemical events leading to shape change, aggregation, dense granule release and eicosanoid generation. Agonist-receptor coupling acts via a guanine-nucleotide-binding protein to activate phospholipase C (PLC) [1], which catalyses the subsequent hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to give two products, inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and sn-1,2-diacylglycerol (DAG). $Ins(1,4,5)P_3$ is thought to mobilise calcium from intracellular stores [2], although cytosolic calcium ($[Ca^{2+}]_i$) is also increased due to an agonist-induced transient permeability of the plasma membrane, allowing extracellular calcium to enter the cells [3]. DAG is a putative endogenous activator of protein kinase C (PKC) [4], a calcium and phospholipid dependent kinase. At least 7 isoenzymes of PKC have been reported [5] of which at least two, namely type II (β chain) and type III (α chain) are thought to exist in human platelets [6].

The role of PKC in platelet activation and in particular eicosanoid generation is still uncertain, despite the availability of potent and specific protein kinase C activators, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [7]. Indeed, phorbol esters and mem-

Abbreviations: PLC, phospholipase C; PIP_2, Phosphatidyl-inositol-4,5-bisphosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; cytosolic calcium, $[Ca^{2+}]_i$; extracellular calcium, $[Ca^{2+}]_e$; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TxB_2, thromboxane B_2; 5-HT, 5-hydroxytryptamine; PAF, platelet activating factor; AA, arachidonic acid; HBT, HEPES buffered Tyrode's solution; 1,2-DHG, 1,2-dihexanyl-sn-diacylglycerol; PLA_2, phospholipase A_2.

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brane permeable DAGs cause both dense granule release [7,8] and platelet aggregation [13] in the absence of any agonist. This led to the hypothesis that a link existed between phosphorylation of a major 40–47 kDa protein substrate of PKC and secretion and aggregation [4,8,10], although a direct relationship has been questioned.

The use of phorbol esters to modulate PKC activity has led to two main theories for the role of PKC in platelet activation. Firstly in platelets which have been permeabilized or treated with ionophores, phorbol esters synergize with increased $[Ca^{2+}]_i$ to potentiate activation [11], demonstrating the requirement for both increased PKC activity and $[Ca^{2+}]_i$ elevation in order to achieve full platelet responses and suggesting a positive role for PKC. In contrast, however, phorbol esters inhibit platelet activation induced by agonists indicating a negative feed back role for PKC [12]. This work has led to the suggestion that PKC may have a dual role in platelet activation.

To further elucidate the role of PKC in platelet activation we have used an alternative experimental approach. We have compared the effect of activation of PKC with the phorbol ester (TPA) with the potent although non-specific protein kinase inhibitor staurosporine [13] on platelet thromboxane (Tx) B_2 generation, 5-hydroxytryptamine (5-HT) release and $[Ca^{2+}]_i$ elevation in platelets stimulated either by a receptor operated stimulus (platelet activating factor, PAF), or by direct cytosolic calcium elevation (ionomycin) or by arachidonic acid (AA) which is metabolised directly into thromboxane. Part of this work has been previously published in abstract form [14].

Materials and Methods

Materials

Prostacyclin (PGI_2) was generously provided by Dr. B.J.R. Whittle, Wellcome Laboratories, U.K. PGI_2 was stored solid and dry at −20°C whilst stock aliquots were dissolved in 0.5 M Tris-HCl (pH 10.5) and were stored at a concentration of 40 μg/40 μl at −20°C. Immediately prior to use an aliquot was thawed and diluted as required in 0.01 M Tris-HCl (pH 10.5) and stored on ice. Fura-2, AM (cell permeant) obtained from Molecular Probes Inc., U.S.A. was stored dry at −20°C. A stock solution at a concentration of 5 mM in anhydrous dimethyl sulfoxide (DMSO) was also stored at −20°C. 5-[^{14}C]Hydroxytryptamine creatinine sulphate (54 mCi/mmol, 50 μCi/ml) was stored at −20°C and [^{32}P]P_i (10 mCi/ml) was stored at 4°C, both were obtained from Amersham International. Thromboxane B_2 standard was obtained from Sigma and [^{3}H]thromboxane B_2 (125 Ci/mmol, 50 nCi/ml) was obtained from New England Nuclear, both were dissolved in ethanol. Rabbit anti-TxB_2 antibody was a gift from Dr F. Carey, ICI, Macclesfield. Platelet activating factor (1-*O*-octadecyl-2-acetyl-sn-glycero-3-phosphocholine) was supplied by Bachem (Bubendorf, Switzerland). A stock solution of 10^{-2} M made up in chloroform : methanol (4 : 1) was stored at −20°C and working solutions (10^{-4} M) were prepared in Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS) buffer containing 2.5 mg/ml bovine serum albumin (BSA). Arachidonic acid obtained from Sigma was dissolved in ethanol at 25 mg/ml and stored under nitrogen at −20°C. The divalent cation ionophore, ionomycin was purchased from Calbiochem and stored at −20°C in DMSO at 5 mg/ml. TPA obtained from Sigma was dissolved (1 mg/ml) in dry acetone and stored at −20°C. 1,2-dihexanyl-sn-glycerol was a kind gift from Dr. L. Garland, Wellcome, it was dissolved in ethanol and stored at −20°C. Staurosporine was purchased from Fluka Chemicals Ltd., U.K., dissolved in DMSO and stored at −20°C.

Methods

Preparation of platelets. Washed rabbit platelets were prepared as described for human platelets by Poll and Westwick [12] using an adaptation of the method of Blackwell et al. [15]. The resulting pellet from this preparation was resuspended in HEPES buffered Tyrode's solution (HBT: 10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 5.5 mM glucose and 0.25% BSA, pH 7.4) and the platelet count adjusted.

Protein phosphorylation. Platelets ($8 \cdot 10^9$/ml) were incubated for 2 h with 200 μCi [^{32}P]P_i/ml, then washed in HBT and the platelet count adjusted to 10^9/ml. Aliquots of platelets (300 μl) were equilibrated at 37°C in the presence of 1 mM extracellular calcium ($[Ca^{2+}]_e$). Platelets were pre-incubated with staurosporine or its vehicle (0.01% DMSO) for 2 min prior to addition of PAF. Activation was terminated at 30 s after PAF addition with 300 μl of 2 × Laemmli SDS-gel sample buffer (100°C). The samples were then boiled for 5 min, and centrifuged at 8000 × *g* to remove any particles. Proteins (100 μg/track) were separated by 10% polyacrylamide SDS-gel electrophoresis [16], protein bands and molecular markers were stained using Coomaissie blue and [^{32}P]P_i phosphorylated bands visualised by autoradiography. The phosphorylated bands were excised and quantitated by liquid scintillation counting.

Determination of $[Ca^{2+}]_i$. Platelets ($8 \cdot 10^8$) were incubated with 2.5 μM Fura-2-acetoxymethyl ester for 30 min at 37°C, the platelet suspension was then washed in HBT and the platelet count adjusted to $2 \cdot 10^8$/ml. After loading and washing the intracellular concentration of Fura-2 was found to be between 10–20 μM. In order to measure changes in $[Ca^{2+}]_i$, fluorescence readings were recorded at 37°C using an Aminco Bow-

man Spectrophotofluorimeter (exc. = 339 nm; emission = 500 nm: 4 nm slot width) fitted with a thermostatted cuvette compartment holder with stirring attachment. 1 ml aliquots of platelets were dispensed into cuvettes and $[Ca^{2+}]_e$ adjusted to 1 mM. Calibration of the calcium-fura-2-fluorescence was carried out as described previously [17,18].

Measurement of % 5-[^{14}C]-HT release. Platelets ($8 \cdot 10^8$/ml) were incubated with 200 nCi/ml 5-[^{14}C]-hydroxytryptamine (5-HT), then washed in HBT and the platelet count adjusted to 2.10^8 platelets/ml. $[Ca^{2+}]_e$ was adjusted to 1 mM and platelets were equilibrated to 37°C prior to the addition of a drug or vehicle control. In order to determine 5-HT release from the platelets, sub-aliquots (400 μl) were withdrawn 3 min after the addition of the agonist into ice-cooled tubes containing 40 μl of 100 mM EDTA and 30 μM indomethacin. 5-HT release was determined at 3 min post-stimulation as there was no further 5-HT release after this time. The samples were centrifuged at $12000 \times g$ and 2×100 μl samples of the supernatant were removed and the amount of ^{14}C-5-HT determined by liquid scintillation spectroscopy. Percentage 5-HT release was determined as described by Holmsen et al. [19].

Measurement of TxB_2 generation. Experiments to determine TxB_2 generation were carried out as for 5-HT release, except using unlabelled platelets. TxB_2 generation was terminated after maximal generation of TxB_2 had occurred (3 min after platelet stimulation) in the same way as described for dense granule release. The TxB_2 content of the supernatant of the samples generated was determined by radio-immunoassay as previously described [20].

Statistics. Except when stated data for the figures is the mean ± S.E. of at least 3 experiments each performed in triplicate. In order to determine the levels of significance between sets of data a paired Student's *t* test was employed. The levels of significance are denoted by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. A Bonferroni correction was employed when necessary [21].

Results

Inhibition of PKC substrate phosphorylation by staurosporine

Staurosporine inhibited 300 nM PAF-induced phosphorylation of the 40–47 kDa PKC substrate in a dose-dependent manner over the range 30–1000 nM with an IC_{50} of 500 nM (Fig. 1). This finding gave a suitable dose range of staurosporine to use in order to investigate the effect of PKC inhibition on platelet functional response.

PKC modulation and PAF-induced platelet activation

Activation of PKC by a 2 min pre-incubation with either 1–30 nM 12-*O*-tetradecanylphorbol 13-actetate (TPA) or 6.8–68 μM of the membrane permeable diacylglycerol, 1,2-dihexanyl-sn-diacylglycerol caused a dose-dependent inhibition of both the rate and maximal height of $[Ca^{2+}]_i$ elevation induced by a submaximal concentration of PAF (3 nM) (Fig. 2A,B). In contrast, 30–1000 nM staurosporine, had no significant effect on either the rate or maximal height of 3 nM PAF-induced $[Ca^{2+}]_i$ elevation (Fig. 2C). Neither TPA, DHG nor staurosporine had any effect on basal levels of $[Ca^{2+}]_i$ which remained at 152 ± 11 nM ($n = 30$).

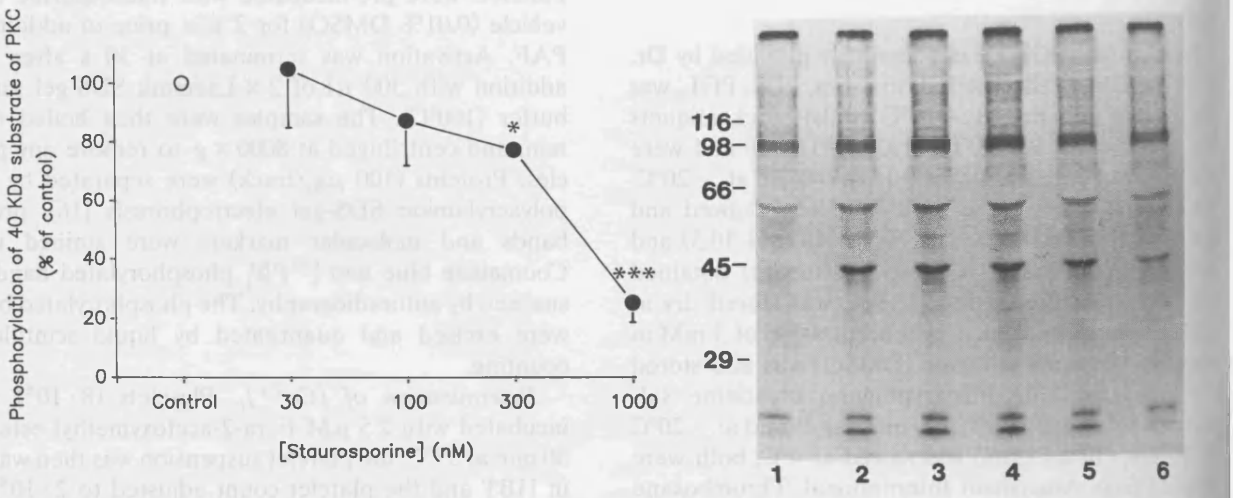
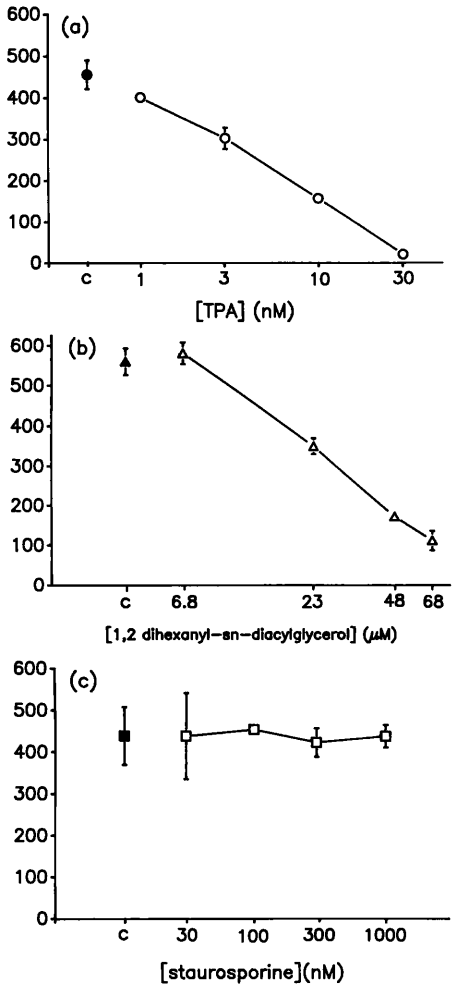


Fig. 1. Inhibition of phosphorylation of the 40–47 kDa substrate of PKC by staurosporine in platelets. Right panel: autoradiograph representing track 1 = phosphorylation in resting platelets, track 2 = 300 nM PAF induced phosphorylation in the presence of staurosporine vehicle (0.01% DMSO) and tracks 3–6 = pretreatment with 30, 100, 300 and 1000 nM SS, respectively, followed by 300 nM PAF stimulation. Left panel: phosphorylation of the PKC substrate as a % of that phosphorylated by 300 nM PAF. Phosphorylation was quantified by scintillation counting of the excised [^{32}P]P; labeled PKC substrate.



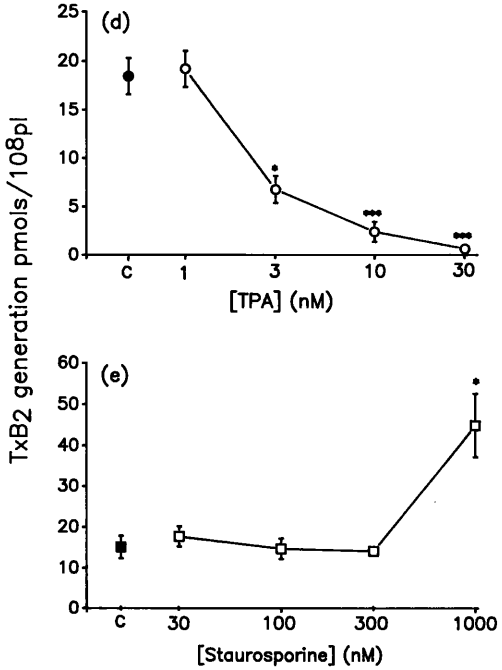


Fig. 2. Effect of PKC modulation on $[Ca^{2+}]_i$ elevation and TxB_2 generation induced by PAF. The left panel demonstrates the effect of a 2 min pre-incubation with the PKC activators (A) TPA and (B) DHG or the PKC inhibitor (C) staurosporine on 3 nM PAF induced $[Ca^{2+}]_i$ elevation. The $[Ca^{2+}]_i$ value was obtained by subtracting the basal pre-PAF value from the peak post-PAF value. The right panel demonstrates the effect of a 2 min pretreatment of (D) TPA and (E) staurosporine on 30 nM PAF induced TxB_2 generation. TxB_2 generation was determined at 3 min after PAF addition.

vas generated in a dose dependent manner in to platelet stimulation over a range of PAF ıtions (data not shown). Only trace amounts < 0.1 pmols/10^8 platelets) were generated in stimulated with 3 nM PAF and therefore a ıal dose of 30 nM PAF was used for the TxB_2 nts. As was found for $[Ca^{2+}]_i$ elevations, pre- ı of PKC with TPA caused a dose dependent of TxB_2 generation (Fig. 2D). Conversely, ition of PKC significantly potentiated TxB_2 n but only at the highest concentration of rine, 1 μM (Fig. 2E). Neither TPA or stau- had any effect on the basal level of TxB_2 n of < 0.1 pmol/10^8 platelets over a 3 min

ependence of TxB_2 generation on $[Ca^{2+}]_i$ was determined by replacing extracellular ($[Ca^{2+}]_e$) with the calcium chelator EGTA Removal of $[Ca^{2+}]_e$ inhibited both $[Ca^{2+}]_i$ and TxB_2 generation induced by PAF sug- ıat TxB_2 generation was dependent on $[Ca^{2+}]_i$

The inhibition of $[Ca^{2+}]_i$ elevation induced by 30 nM TPA was dose-dependently abrogated by staurosporine in 3 nM PAF stimulated platelets (Fig. 4), indicating that the inhibition of $[Ca^{2+}]_i$ elevation by TPA and 1,2-DHG was mediated by PKC.

PKC modulation and ionomycin-induced platelet activation

In contrast to its effect on $[Ca^{2+}]_i$ elevation induced by PAF, TPA had no effect on $[Ca^{2+}]_i$ elevation in platelets stimulated with a submaximal concentration (5 μM) of ionomycin (Fig. 5A). Inhibition of PKC with staurosporine also had no effect on ionomycin induced $[Ca^{2+}]_i$ elevation (Fig. 5B). Addition of 2.8 μM indomethacin had no effect on ionomycin stimulated $[Ca^{2+}]_i$ elevation either in the presence or absence of TPA or staurosporine (not shown).

TxB_2 generation in ionomycin stimulated platelets was, however, modified by both PKC activation and inhibition. PKC activation with TPA (1–30 nM) caused a dose dependent potentiation of TxB_2 generation (Fig. 5C), whereas PKC inhibition with staurosporine

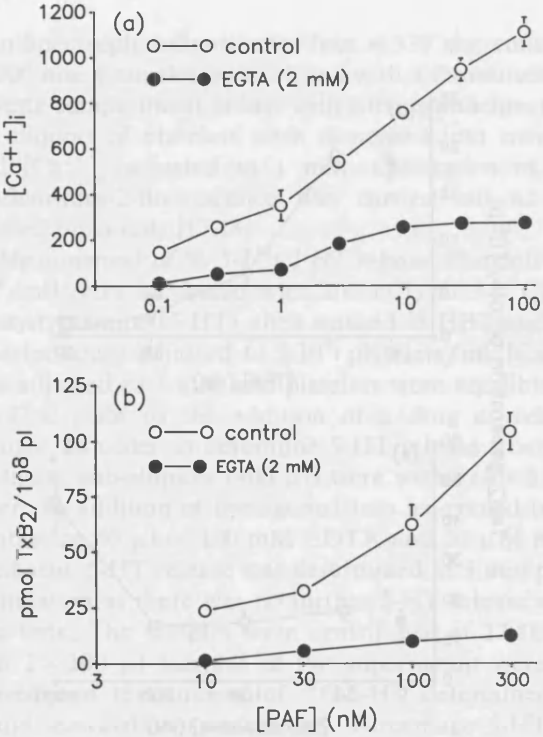


Fig. 3. The effect of replacing 1 mM extracellular calcium with 1 mM EGTA on PAF-induced (A) $[Ca^{2+}]_i$ elevation and (B) TxB_2 generation. The top panel shows peak $[Ca^{2+}]_i$ elevations after subtraction of the basal pre-PAF value, and the lower panel TxB_2 generation determined 3 min after the addition of PAF. Open circles denote the presence of 1 mM $[Ca^{2+}]_e$ and closed circles 1 mM $[EGTA]_e$.

caused a dose dependent inhibition of TxB_2 generation (Fig. 5D).

Although 5 μM ionomycin was a submaximal con-

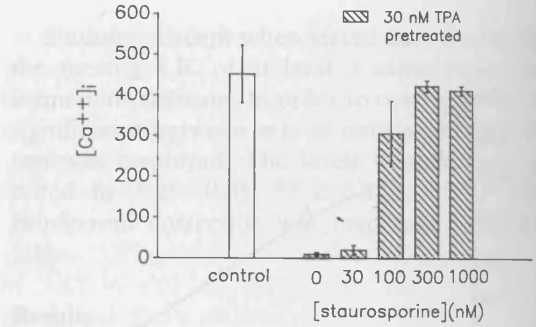


Fig. 4. Reversal of TPA-induced inhibition of $[Ca^{2+}]_i$ elevation by staurosporine in 3 nM PAF-stimulated platelets. Staurosporine was added 2 min before TPA (30 nM) which was added 2 min prior to stimulation with 3 nM PAF. $[Ca^{2+}]_i$ is the value obtained by subtracting the basal pre-PAF value from the maximal post-PAF value.

centration for cell functional responses it was important to establish that this concentration of ionomycin was not causing non-specific cell permeabilization or cell lysis, therefore we examined the supernatant of ionomycin stimulated cells for lactate dehydrogenase activity, a marker for cell damage and we also examined for low molecular weight molecule leakage by testing for leakage of Fura-2 from the cells. At 5 μM ionomycin, there was no lactate dehydrogenase activity in the supernatant nor did any Fura-2 leak from the cells into the extracellular medium until a concentration of above 50 μM ionomycin was added to the platelets.

PKC modulation and arachidonic acid-induced platelet activation

In agreement with the findings for PAF stimulated

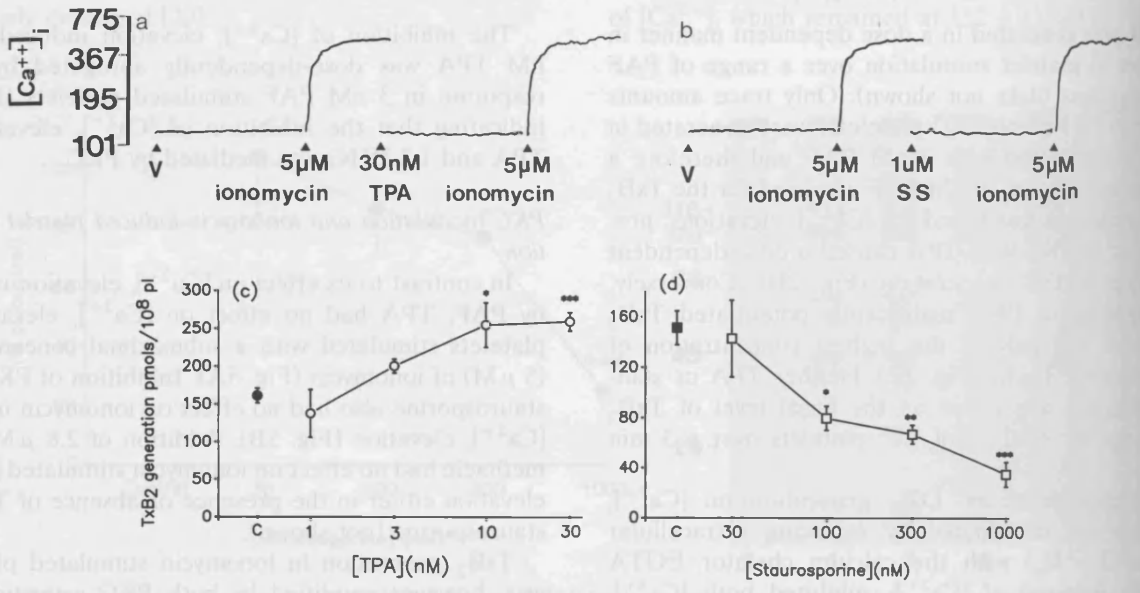


Fig. 5. PKC modulation on 5 μM ionomycin induced $[Ca^{2+}]_i$ elevation and TxB_2 generation. The traces in the upper panel represent the fluorescent output of fura-2 loaded platelets which have been pretreated for 2 min with (A) 30 nM TPA or (B) 1 μM staurosporine and stimulated with ionomycin. The lower panel is the effect of pretreatment with (C) TPA or (D) staurosporine (SS) on TxB_2 generation induced by ionomycin. TxB_2 generation was determined 3 min post stimulation.

platelets, TPA caused a dose dependent inhibition of $[Ca^{2+}]_i$ elevation whilst staurosporine had no effect on $[Ca^{2+}]_i$ elevation in platelets stimulated with arachidonic acid (AA) (results not shown). Neither PKC activation nor inhibition had any effect on TxB_2 generation in platelets stimulated with AA (Fig. 6A,B). TxB_2 generation stimulated by PAF, ionomycin or AA could be completely inhibited by the addition of 2.8 μM indomethacin. A concentration of 30 μM arachidonic acid was found to be submaximal for both $[Ca^{2+}]_i$ elevation and TxB_2 generation. It was important to establish 30 μM AA was not causing non-specific cell permeability or lysis, therefore we examined the supernatant of AA stimulated platelets for lactate dehydrogenase, a marker for cell damage and for Fura-2 leakage from the cell upon AA stimulation. There was no lactate dehydrogenase activity in the supernatant nor had any Fura-2 leaked into the extracellular medium of platelets stimulated with 30 μM AA.

PKC modulation on PAF and ionomycin-induced 5-HT release

Washed platelets released 5-HT from their dense granules in response to stimulation with either PAF or ionomycin (Fig. 7). Activation of PKC with TPA alone also caused a small amount of release, with up to 6% at 30 nM TPA. In platelets stimulated with submaximal PAF (3 nM) pre-activation of PKC with 1–30 nM TPA had no significant effect on 5-HT release (Fig. 7A). Staurosporine, however, inhibited 5-HT release with an IC_{50} of 800 nM, causing up to 70% inhibition with 1 μM staurosporine (Fig. 7B).

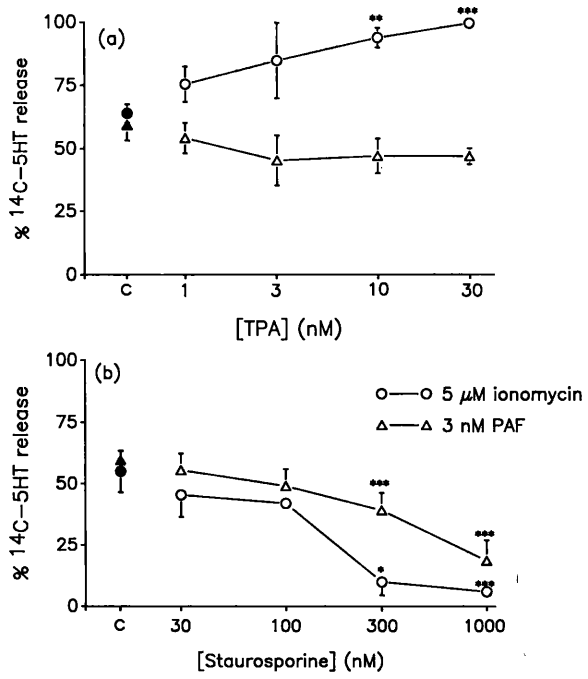


Fig. 7. Effect of (A) TPA or (B) staurosporine on 3 nM PAF (triangles) or 5 μM ionomycin (circles) induced 5-HT release. Results are given as a % of total possible 5-HT release. The closed symbols denote release of 5-HT by PAF or ionomycin in the presence of TPA vehicle or staurosporine vehicle. % 5-HT release was measured 3 min after PAF or ionomycin stimulation.

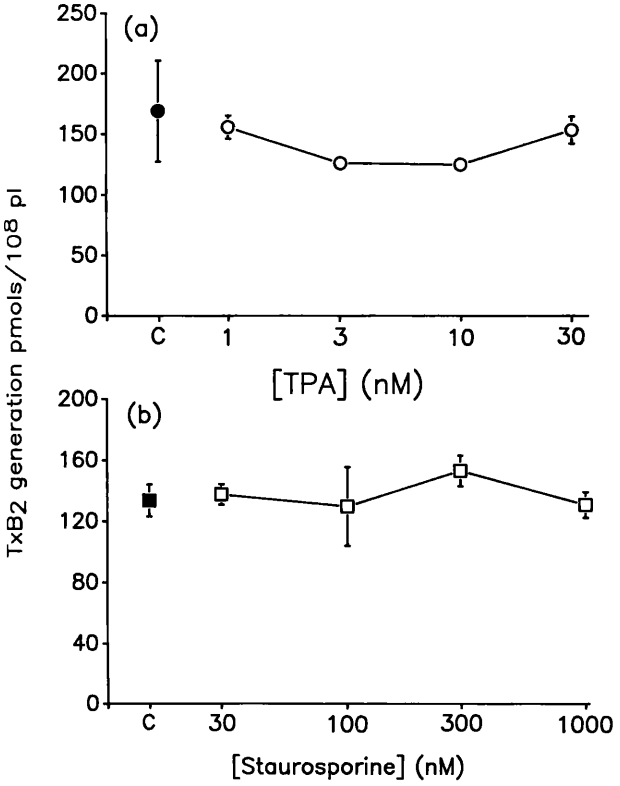


Fig. 6. PKC modulation on arachidonic acid (AA)-induced TxB_2 generation. Platelets were pretreated for 2 min with either (A) TPA or (B) staurosporine and stimulated with 30 μM arachidonic acid. TxB_2 generation was measured at 3 min post-stimulation.

In ionomycin stimulated platelets however, TPA caused a significant potentiation of 5-HT release with 100% release at 30 nM TPA (Fig. 7A). This potentiation was not due to the potentiation of TxB_2 generation by TPA in ionomycin stimulated platelets, as 2.8 μM indomethacin had no effect on TPA potentiated ionomycin-induced 5-HT release. In direct contrast staurosporine caused a significant inhibition of 5-HT release with an IC_{50} of 150 nM, and produced almost 100% inhibition at only 300 nM SS (Fig. 7B). Staurosporine was therefore a weaker inhibitor of 5-HT release in platelets stimulated via a receptor operated mechanism (PAF) than in platelets directly activated via calcium elevation (ionomycin). Experiments measuring 5-HT release were carried out in both platelets loaded with the fluorescent dye Fura-2 used to measure $[Ca^{2+}]_i$ elevation and in platelets not loaded with Fura-2. Fura-2 loading had no effect on 5-HT release in either PAF or ionomycin stimulated platelets, indicating that Fura-2 was not chelating cytosolic calcium to an extent where calcium dependent functional responses were suppressed.

Discussion

The most abundant eicosanoid produced in platelets is the cyclo-oxygenase product TxA_2 which is both a platelet agonist, a vasoconstrictor and is also involved in platelet-vessel wall interactions [22]. TxA_2 is synthesised from AA via the cyclo-oxygenase pathway and TxA_2 synthetase [23]. AA is produced from two sources, the most important of which is thought to be its release from membrane phospholipids such as phosphatidylcholine and phosphatidlyinositol by the action of the calcium and phospholipid dependent enzyme phospholipase A_2 (PLA_2). The second source of AA is from the action of diacylglycerol and monoacylglycerol lipase on sn-1,2 DAG produced by the hydrolysis of PIP_2 by PLC [24]. By studying both activation and inhibition of PKC, we have demonstrated that PKC is one of the factors capable of modulating TxA_2 generation, but that depending on the method of platelet stimulation, it can play either an inhibitory or a potentiatory role.

It is well established that pre-activation of PKC in platelets stimulated with various agonists causes an inhibition of $[Ca^{2+}]_i$ elevation (eg Ref. 25). In agreement with this we have demonstrated that pretreatment with either the phorbol ester TPA, or the membrane permeable diacylglycerol, 1,2 dihexanyl-sn-diacylglycerol causes inhibition of subsequent $[Ca^{2+}]_i$ elevation in platelets stimulated by PAF. This negative feedback pathway is thought to be partly due to the PKC activation inhibiting PLC activity, and therefore the production of the second messenger $Ins(1,4,5)P_3$ which is believed to mobilise intracellular calcium stores [2], and in combination with its metabolite inositol 1,3,4,5-tetrakisphosphate regulate second messenger operated calcium channels [26]. Pre-activation of PKC also increases the rate of $Ins(1,4,5)P_3$ dephosphorylation to inactive $I(1,4)P_2$ [27,28]. Indeed it has been suggested that 5′-phosphomonoesterase is a substrate of PKC [28]. Activating PKC by increasing endogenous DAG by the use of the DAG kinase inhibitor R44949 also inhibits $[Ca^{2+}]_i$ elevation in agonist stimulated platelets [29], suggesting that endogenously activated PKC is exerting a similar mechanism to that seen with exogenously activated PKC.

We have demonstrated that by replacing $[Ca^{2+}]_e$ with the calcium chelator EGTA, PAF-induced $[Ca^{2+}]_i$ elevation and also TxB_2 generation were significantly inhibited. As PLA_2 is a calcium-dependent enzyme inhibition of $[Ca^{2+}]_i$ elevation may lead to a reduction in its activity and consequently inhibition of AA production. In addition, inhibition of PLC activity by PKC activation will inhibit DAG production which is also a source of AA and therefore TxB_2 [24]. This suggests that inhibition of $[Ca^{2+}]_i$ elevation by TPA may be responsible for the subsequent inhibition of TxB_2 generation observed in our study. This may not be true of all agonists, however, as collagen has been shown to activate PLA_2 independently of changes in $[Ca^{2+}]_i$ [18]. Alternatively, there are now several lines of evidence which suggest PLA_2 may be directly coupled to membrane receptors via GTP-binding proteins [30] indicating activity of PLA_2 is not subsequent to PLC activation but a separate event. One way PLC may be is modulated by PKC is via phosphorylation of the G-proteins that link PLC to membrane receptors. It should not be ruled out, therefore, that PKC is exerting its effect over PLA_2 in the same way. It is more likely however, that PLA_2 activity is modified by a number factors. Inhibition of PKC using the non-specific kinase inhibitor staurosporine had no effect on either the rate or maximal elevation of $[Ca^{2+}]_i$ induced by sub-maximal levels of PAF (3 nM). However, in agreement with Watson et al. [10], we found that inhibition of PKC with 1 μM staurosporine caused an increase in duration of calcium elevation. This finding supports previous studies where activation of PKC increases the rate of decline of agonist-induced $[Ca^{2+}]_i$ [31] and suggests a role for PKC in calcium homeostasis. In direct contrast to the effect of PKC activation, staurosporine potentiated TxB_2 generation supporting the theory that endogenously activated PKC exerts a negative feedback over PLC and in addition linking changes in $[Ca^{2+}]_i$ to those in TxB_2 generation. Recently several inhibitors with an improved specificity for PKC have been developed, namely Ro 31-7549/001 and Ro 31-8220/002 [32]. We have compared the effect of these inhibitors to staurosporine on PAF-induced platelet functional responses, and have demonstrated that like staurosporine the selective inhibitors of PKC have no effect on maximal elevation of $[Ca^{2+}]_i$ whilst increasing the duration of $[Ca^{2+}]_i$ elevation and TxB_2 generation (unpublished observation). This would indicate that the effects observed with staurosporine are likely to be due to its inhibitory effect on PKC and not other kinases. Indeed we have also demonstrated that inhibition of tyrosine kinase with the selective inhibitor genistein [33] causes an inhibition of both maximal $[Ca^{2+}]_i$ elevation and TxB_2 generation which is in direct contrast to the effects of staurosporine (unpublished observation).

A direct increase in $[Ca^{2+}]_i$ in combination with PKC activation has been found to act synergistically to increase platelet activation [11]. Our results confirm and extend these observations by demonstrating that whilst pre-incubation with the PKC activator TPA has no effect on maximal $[Ca^{2+}]_i$ elevation induced by ionomycin, both 5-HT release and TxB_2 generation are significantly potentiated. Halenda et al. [34] demonstrated that phorbol esters do not interfere with the reincorporation of AA into phospholipids, therefore the potentiation of TxB_2 found with TPA cannot be attributed to modulation of phospholipid reincorpora-

tion. Staurosporine also has no effect on maximal $[Ca^{2+}]_i$ elevation induced by ionomycin, however in contrast to PKC activation both 5-HT release and TxB_2 generation are significantly inhibited. Therefore in ionomycin-stimulated platelets activation of PKC potentiates functional responses whilst inhibition suppresses them. Although ionomycin has been demonstrated to liberate arachidonic acid directly, indomethacin had no effect on either ionomycin-induced $[Ca^{2+}]_i$ elevation nor 5-HT release in this study indicating the release of AA by ionomycin is not influencing these responses. Thus the effect of PKC modulation in platelets stimulated with ionomycin differs greatly from that in receptor-activated platelets, and makes the use of ionophores together with PKC activators a poor model to use to investigate the role of endogenously activated PKC.

In ionomycin-stimulated platelets therefore, $[Ca^{2+}]_i$ elevation is not affected by PKC modulation but TxB_2 generation is affected. Indeed it is thought that in ionomycin stimulated platelets, platelet activation is independent of PLC activity, with all PLC activation being due entirely to the generation of cyclo-oxygenase products [35]. It maybe, therefore, that an influx of calcium by ionomycin directly activates PLA_2, to form AA, production of which may be directly modulated by PKC-induced phosphorylation at the enzyme site. The form this modulation may take is not known, but it is possible that PKC may interact directly with PLA_2 or a coupled G-protein, or may act to remove the inhibitory effect on PLA_2 of an intermediary protein (eg. an annexin) [36]. Indeed a 35–36 kDa protein with a similar pI to that reported for annexin is phosphorylated in platelets stimulated with ionophore and the phorbol ester, PMA [37]. It has also been shown that annexins are phosphorylated in vitro by PKC [38]. Since neither TPA nor staurosporine were found to modify arachidonic acid-induced TxB_2 generation this indicates that the site of PKC modulation is unlikely to be the cyclo-oxygenase pathway or TxB_2 synthetase.

PKC activation and inhibition respectively potentiate and inhibit dense granule release in ionomycin stimulated platelets, indicating a modulatory role for PKC in ionomycin-induced 5-HT release. In contrast, in PAF-stimulated platelets activation of PKC with TPA had no effect on 5-HT release, whilst compared to its effects in ionomycin stimulated platelets, staurosporine caused only a weak inhibition. A good correlation is thought to exist between the phosphorylation state of the major 40–47 kDa protein substrate of PKC and 5-HT release [8]; indeed we and others have found that PKC activation with TPA alone causes a release of 5-HT [8]. Our findings indicate that 5-HT release, as opposed to TxB_2 generation, may have a closer affinity to the phosphorylation state of PKC substrates. As the effect of PKC inhibition is the same in ionomycin as in PAF-stimulated platelets this may indicate that PLC is not involved. However, staurosporine is a weaker inhibitor of PAF-induced 5-HT release than ionomycin-induced 5-HT release, indicating perhaps that PKC is less important in modulating agonist-induced dense granule release than modulating ionomycin-induced dense granule release.

In conclusion therefore, PKC is able to modulate thromboxane generation in both PAF and ionomycin-stimulated platelets, but with opposite effects. The results suggest that in ionomycin-activated platelets (where elevation of $[Ca^{2+}]_i$ elevation is not effected by PKC activation or inhibition) the activation state of PKC is directly related to TxB_2 generation. In PAF-stimulated platelets however, the effect of PKC activation/inhibition on $[Ca^{2+}]_i$ elevation appears to be important in modulating TxB_2 generation although additional factors as discussed may also play an important role. In addition the findings of this study also demonstrate that a different type of relationship exists between PKC and dense granule release than between PKC and TxB_2 generation in PAF-stimulated platelets.

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Selective inhibition of protein kinase C

Effect on platelet-activating-factor-induced platelet functional responses

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The role of protein kinase C (PKC) in platelet-activating-factor (PAF)-induced platelet activation was examined by using two selective inhibitors of PKC, namely Ro 31-7549/001 and Ro 31-8220/002. Both inhibitors dose-dependently inhibited PAF-induced phosphorylation of the major 40–47 kDa protein substrate of PKC, with 50% inhibition at 4.5 μM-Ro 31-7549/001 and 0.7 μM-Ro 31-8220/002. Inhibition of PKC had no effect on maximal elevation of intracellular Ca^{2+} $[Ca^{2+}]_i$ produced by either a high or a low dose of PAF, but significantly increased the duration of the Ca^{2+} signal and the thromboxane B_2 (TxB_2) generation in high-dose PAF-stimulated platelets. The inhibitors also abrogated the effect of the PKC activator phorbol 12-myristate 13-acetate on PAF-induced $[Ca^{2+}]_i$ elevation. Sub-maximal PAF-induced dense-granule release and platelet aggregation were dose-dependently inhibited by Ro 31-7549/001 and Ro 31-8220/002. The findings suggest that endogenously activated PKC holds a bifurcating role in PAF-activated platelets, negatively affecting duration of both $[Ca^{2+}]_i$ and TxB_2 generation, and positively influencing dense-granule release and aggregation.

INTRODUCTION

Protein kinase C (PKC) is thought to hold a pivotal role in agonist-initiated signal transduction in the platelet (Nishizuka, 1984). Agonist-receptor-coupled platelet activation induces the hydrolysis of phosphoinositides, producing the two signal molecules *sn*-1,2-diacylglycerol (DAG) and Ins (1,4,5)P_3 (MacIntyre & Pollock, 1983; Agranoff *et al.* 1983). DAG is the putative endogenous activator of PKC (Lapetina *et al.*, 1985), a family of Ca^{2+}-and-phospholipid-dependent isoenzymes, which phosphorylate target proteins. In platelets their major substrate is a 40–47 kDa protein of unknown function (Tyers *et al.*, 1988). Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) activate PKC by increasing its affinity for Ca^{2+} and phospholipid (Castagna *et al.*, 1982). As a consequence, phorbol esters have been widely used to try to establish the role of PKC in signal transduction. However, owing to the use of permeabilized platelet preparations in many of these studies (Halenda *et al.*, 1985; Kaibuchi *et al.*, 1983; Mobley & Tai, 1985) and also the differences between PMA and endogenous DAG, including both their permeability and longevity (Bazzi & Nelsestuen, 1989), these studies are difficult to relate to the physiological situation. On the other hand, an inhibitor of PKC would allow the role of endogenously activated PKC to be examined.

Some of the earliest PKC inhibitors described include chlorpromazine, dibucaine and other phospholipid-interacting drugs (Mori *et al.*, 1980), including polymyxin B (Mazzei *et al.*, 1982), which inhibit PKC by competing with phospholipid. Later it was found that members of the isoquinoline sulphonamide family, particularly H-7, were capable of inhibiting PKC by competing with ATP for its binding site on PKC (Hidaka *et al.*, 1984; Yamamoto & Hidaka, 1984). In 1985 the non-steroidal triphenylacrylonitrile (TPE) tamoxifen used in the treatment of breast cancer was shown to inhibit PKC (O'Brian *et al.*, 1985), and more recently was shown to be relatively selective for PKC (Spacey *et al.*, 1990). Depending on the structure of the TPE, it inhibits PKC either at the regulatory or the catalytic domain (Bignon *et al.*, 1989). In order for the role of PKC to be unambiguous, however, an inhibitor would need to be both potent and selective for PKC above other protein kinases. Until the discovery of staurosporine (Tamaoki *et al.*, 1986) and members of the same family, including K252a (Yamada *et al.*, 1987) and UCN01 (Ruegg & Burgess, 1989), all inhibitors of PKC had low potency and, except tamoxifen, were non-selective. Staurosporine and related compounds were found to be potent inhibitors of PKC, but because they inhibited by competing with ATP for the ATP co-substrate site common to all protein kinases, they had limited selectively for PKC (Ruegg & Burgess, 1989). Since the introduction of staurosporine, other PKC inhibitors, including amino acrides (Hannun & Bell, 1988; Smal *et al.*, 1989), pseudo-substrate analogues of PKC (Ricouart *et al.*, 1989) and calphostin C (Kobayashi *et al.*, 1989), have been produced which are thought to inhibit PKC at the catalytic and regulatory sites, at the substrate-binding site and at the regulatory domain respectively.

Several potent and selective inhibitors of PKC have been described (Davis *et al.*, 1989). In this study we have used two of these, namely Ro 31-7549/001 and Ro 31-8220/002, which, although structured on staurosporine and inhibiting PKC by competing with ATP for the co-substrate site, have been demonstrated to be selective for PKC over both protein kinase A and Ca^{2+}/calmodulin dependent kinase (Davis *et al.*, 1989). We have used these two compounds in order to examine the role of endogenously activated PKC in the signal-transduction pathway in platelet-activating-factor (PAF)-stimulated rabbit platelets.

MATERIALS AND METHODS

Materials

Prostacyclin was generously provided by Dr. B. J. R. Whittle, Wellcome Laboratories, Beckenham, Kent, U.K. Stock samples were dissolved in 0.5 M-Tris/HCl (pH 10.5) at a concentration of

Abbreviations used: PKC, protein kinase C; DAG, *sn*-1,2-diacylglycerol; PMA, phorbol 12-myristate 13-acetate; TPE, triphenylacrylonitrile; PAF, platelet-activating factor; HBT, Hepes-buffered Tyrode's solution; TxB_2, thromboxane B_2; $[Ca^{2+}]_i$, intracellular Ca^{2+}; 5-HT, 5-hydroxytryptamine; IC_{50}, concn. giving 50% inhibition; PLC, phospholipase C.

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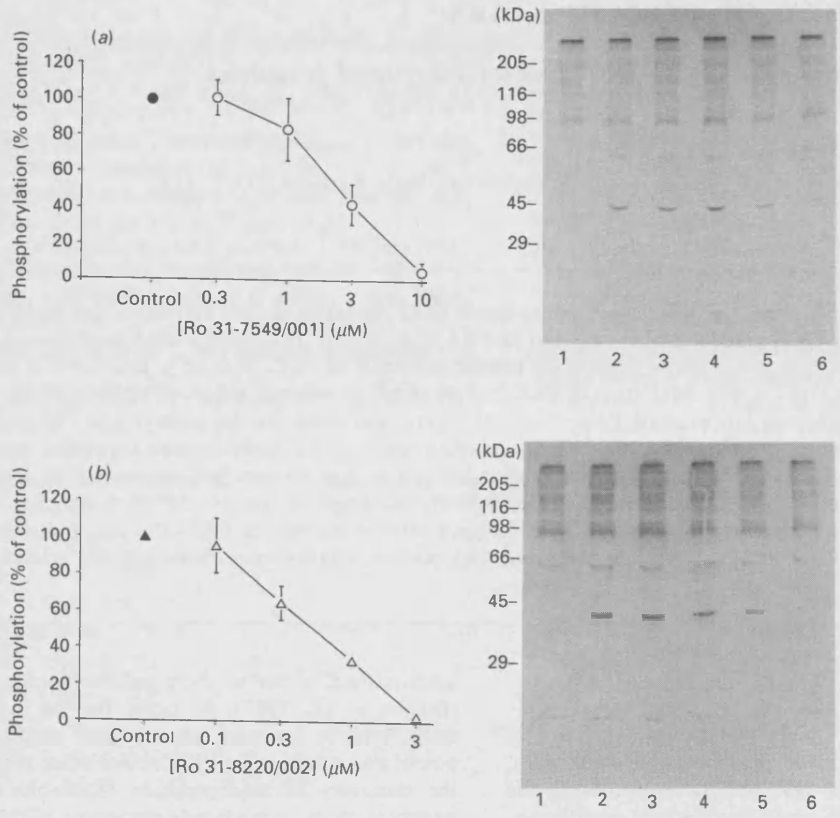

Fig. 1. Inhibition of PAF-induced phosphorylation of the 40–47 kDa substrate of PKC by Ro 31-7549/001 (*a*) and Ro 31-8220/002 (*b*)

The right panels in (*a*) and (*b*) are autoradiographs representing: in track 1, phosphorylation in resting platelets; in track 2, 300 nM-PAF-induced platelet phosphorylation in the presence of the respective PKC inhibitor vehicle; and in tracks 3–6 respectively, either a 20 min preincubation with 0.3 μM-, 1 μM-, 3 μM-, 10 μM-Ro 31-7549/001 (*a*) or 0.1 μM-, 0.3 μM-, 1 μM-, 3 μM-Ro 31-8220/002 (*b*), followed by 300 nM-PAF stimulation for 30 s. On the left, phosphorylation was quantified by liquid-scintillation counting of the excised $[^{32}P]P_i$-labelled PKC substrate. The data are expressed as phosphorylation of the PKC substrate, as a percentage of that phosphorylated by 300 nM-PAF alone (●; control), and each point is the mean ± S.E.M. of three separate experiments.

40 μg/40 μl. Working samples were diluted in 0.01 M-Tris/HCl (pH 10.5) and kept on ice. 5-Hydroxy[^{14}C]tryptamine (5-H[^{14}C]T) creatinine sulphate (54 mCi/mmol; 50 μCi/ml) and $[^{32}P]P_i$ (5 mCi/ml) were purchased from Amersham International. X-ray film was purchased from Fuji. Fura-2 AM (acetoxymethyl ester; cell-permeant) was obtained from Molecular Probes, Eugene, OR, U.S.A., and a stock solution at a concentration of 5 mM in anhydrous dimethyl sulphoxide was stored at −20 °C. Thromboxane B_2 (TxB_2) standard was obtained from Sigma, $[^3H]TxB_2$ (125 Ci/mmol; 50 nCi/ml) from New England Nuclear and anti-(rabbit TxB_2) antibody was generously supplied by Dr. F. Carey, ICI, Macclesfield, U.K. PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was supplied by Bachem (Bubendorf, Switzerland). PMA was obtained from Sigma, dissolved in dry acetone and stored at −20 °C. PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002, were kindly given by Dr. J. S. Nixon, Roche, Welwyn, Herts., U.K. Both PKC inhibitors were dissolved at 10 mM in dimethyl sulphoxide, and further dilutions were made in Hepes-buffered Tyrode's solution (HBT).

Methods

Preparation of platelets. Washed rabbit platelets were prepared as described for human platelets by Poll & Westwick (1986*a*) by a modification of an original method by Blackwell *et al.* (1982). The resulting platelet pellet from this preparation was suspended in HBT (10 mM-Hepes, 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 0.5 mM-Na_2HPO_4, 5.5 mM-glucose and 0.25% BSA, pH 7.4) and the platelet count adjusted. All experiments were carried out at 37 °C in the presence of 1 mM extracellular Ca^{2+}.

Protein phosphorylation. Platelets (8×10^9/ml) were incubated for 2 h with 200 μCi of $[^{32}P]P_i$/ml of platelets, washed in HBT, and the platelet count was adjusted to 10^9/ml. Samples of platelets (300 μl; 10^9 cells/ml) were preincubated with a dose range of the PKC inhibitors or their vehicle for 20 min before activation with 300 nM-PAF. Activation was terminated with 300 μl of 2 × Laemmli (1970) SDS reducing gel sample buffer (100 °C) 30 s after PAF addition. The samples were then boiled for 5 min, and centrifuged at 8000 *g* for 2 min to remove any particles. Proteins (100 μg/track) were separated by SDS/PAGE (10% gel), and protein bands and molecular markers were stained with Coomassie Blue. The gels were then dried and $[^{32}P]$phosphorylated bands were detected by autoradiography. The phosphorylated bands were excised and quantified by liquid-scintillation counting.

Determination of intracellular Ca^{2+} concn. ($[Ca^{2+}]_i$). Platelets were loaded with 2.5 μM fura-2 AM for 30 min at 37 °C, the platelet suspension was then washed in HBT to remove excess fura-2 AM, and the platelet count adjusted to 2×10^8/ml. In order to measure the changes in $[Ca^{2+}]_i$, fluorescence readings were recorded with an Aminco–Bowman spectrophotofluorimeter (excitation at 339 nm, emission at 500 nm; 4 nm slit width) fitted with a thermostatically controlled cuvette compartment holder with stirring attachment. The Ca^{2+}/fura-2-fluorescence was calibrated as previously described (Grynkiewicz

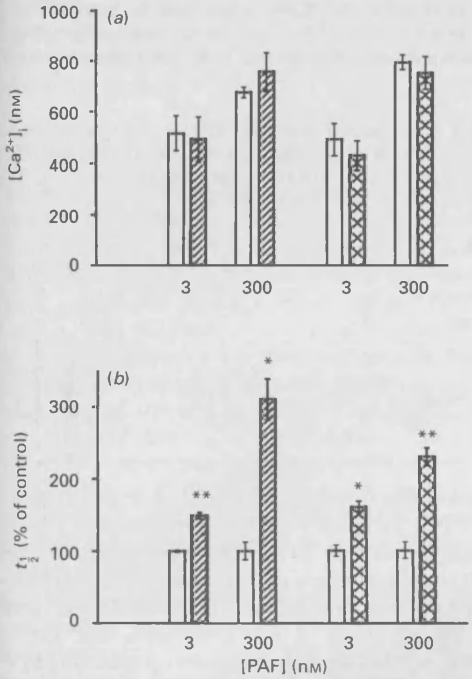

Fig. 2. Effect of the PKC inhibitors on maximal $[Ca^{2+}]_i$ elevation (*a*) and the duration of $[Ca^{2+}]_i$ elevation (*b*) in 3 nM- and 300 nM-PAF-stimulated platelets

Platelets were incubated with 10 μM-Ro 31-7549/001 ▨, 3 μM-Ro 31-8220/002 (⊠) or their appropriate vehicle (□, control; < 0.1% dimethyl sulphoxide) 20 min before addition of PAF. $[Ca^{2+}]_i$ is the maximal elevation of cytosolic Ca^{2+} after subtracting the basal pre-PAF value. $t_{\frac{1}{2}}$ is the time for Ca^{2+} to decrease to half-maximal above basal levels; results are expressed as percentages of the control $t_{\frac{1}{2}}$.

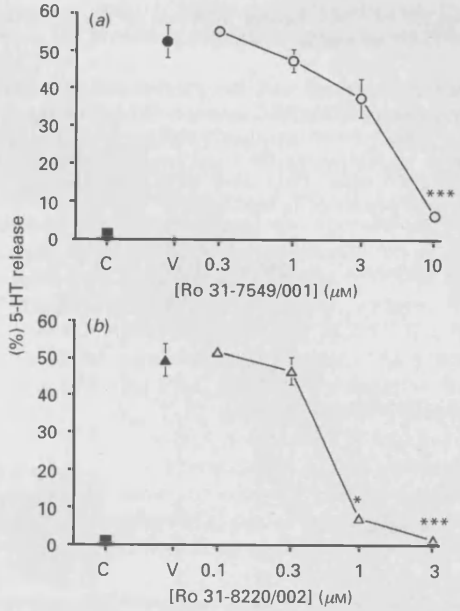

Fig. 3. Effect of the PKC inhibitors on dense-granule release induced by PAF

Platelets were treated for 20 min with Ro 31-7549/001 (○) or Ro 31-8220/002 (△) before activation with 3 nM-PAF. The percentage 5-HT release induced by the highest concentration of each PKC inhibitor alone (C) is shown as ■ in each panel, and the effect of the PKC-inhibitor vehicle (V) on 3 nM-PAF-induced granule release is shown as ● and ▲ for Ro 31-7549/001 and Ro 31-8220/002 respectively. Dense-granule release was terminated 3 min after addition of PAF.

et al., 1985; Pollock *et al.*, 1986). Addition of the PKC inhibitors or their vehicle was made 20 min before stimulation with PAF.

Measurement of TxB_2 generation. This was done with 1 ml samples of platelets (2×10^8/ml), and PKC inhibitor/vehicle was added 20 min before PAF. To determine TxB_2 generation, sub-samples of platelets (400 μl) were withdrawn at 3 min into 40 μl of ice-cold 100 mM-EDTA and 30 μM-indomethacin. The samples were centrifuged for 2 min at 12000 *g*, the supernatant was removed and the TxB_2 content was determined by radioimmunoassay (Johnston *et al.*, 1984).

Measurement of 5-H[^{14}C]T release. Platelets (8×10^8/ml) were incubated with 200 nCi of 5-H[^{14}C]T/ml, then washed in HBT, and the platelet count was adjusted to 2×10^8/ml. Addition of a PKC inhibitor or its vehicle control was made 20 min before stimulation with 3 nM-PAF. The 5-HT release from the platelets was terminated as described for termination of TxB_2 generation. After the samples were centrifuged two 100 μl samples of the supernatant were removed and the amount of 5-H[^{14}C]T was determined by liquid-scintillation spectroscopy. Percentage 5-HT release was determined as previously described (Holmsen & Dangelmaier, 1989).

Platelet aggregation. This was continually monitored by a twin-channel Ca^{2+} aggregometer, and the results were expressed as a percentage increase in light transmission. Samples of platelets (2×10^8/ml) were pretreated with the PKC inhibitors for 20 min before addition of PAF. The 100% light transmission was that produced by diluting the platelets to 2×10^7/ml. Platelets were continually stirred throughout experimentation at 900 rev./min.

Statistics. Data are means ± S.E.M. of at least three experiments each performed in triplicate. To determine the levels of significance between sets of data, a paired Student's *t* test was employed. The levels of significance are denoted by $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. The Bonferroni correction was applied where necessary (Wallenstein *et al.*, 1980).

RESULTS

Inhibition of phosphorylation of the major substrate of PKC

A 20 min preincubation with the selective PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002 inhibited 300 nM-PAF-induced phosphorylation of the 40–47 kDa major PKC substrate in a dose-dependent manner (Figs. 1*a* and 1*b*). Ro 31-7549/001 inhibited phosphorylation with an IC_{50} (concn. giving 50% inhibition) of 2.5 μM, and Ro 31-8220/002 inhibited phosphorylation with an IC_{50} of 0.45 μM.

Effect of PKC inhibition on PAF-induced functional responses

$[Ca^{2+}]_i$ elevation. The effect of inhibition of PKC on $[Ca^{2+}]_i$ elevation was investigated by using platelets stimulated with both sub-maximal (3 nM) and maximal (300 nM) doses of PAF (Fig. 2). Doses of 10 μM-Ro 31-7549/001 and 3 μM-Ro 31-8220/002 were used, as these concentrations were found to inhibit PKC activation by > 90% (Fig. 1). Preincubation with either Ro 31-7549/001 or Ro 31-8220/002 for 20 min had no effect on maximal $[Ca^{2+}]_i$ elevation induced by either 3 nM- or 300 nM-PAF; however, both inhibitors significantly increased the duration of the Ca^{2+} signal (Fig. 2). Moreover, whereas in 300 nM-PAF-stimulated platelets inhibition of PKC caused up to a 180% increase in duration of the Ca^{2+} signal, it only caused about a 50% increase with 3 nM-PAF (Fig. 2*b*).

Reversal of PMA-induced PKC activation by using Ro 31-7549/001 and Ro 31-8220/002. The ability of the PKC inhibitors

Table 1. Abrogation of PMA-induced inhibition of $[Ca^{2+}]_i$ elevation by Ro 31-7549/001 and Ro 31-8220/002

Platelets were preincubated with the PKC inhibitors or vehicle 20 min before addition of the PKC activator PMA (30 nM) and then incubated for 2 min before stimulation with 3 nM-PAF. $[Ca^{2+}]_i$ is the value obtained by subtracting the basal pre-PAF value from the maximal post-PAF value. Data were taken from three separate experiments each performed in triplicate.

PKC inhibitor (μM)	PMA (nM)	PAF (nM)	$[Ca^{2+}]_i$ (nM)
–	–	3	432±33
–	30	3	12±5
Ro 31-7549/001			
0.3	30	3	24±10
1	30	3	93±41
3	30	3	562±22
10	30	3	524±53
Ro 31-8220/002			
0.1	30	3	5±4
0.3	30	3	42±8
1	30	3	450±86
3	30	3	441±47

to abrogate the effect of PKC activation was studied by investigating their ability to reverse the effect of the PKC activator PMA on PAF-induced $[Ca^{2+}]_i$ elevation (Table 1). Activation of PKC with 30 nM-PMA inhibited $[Ca^{2+}]_i$ elevation in PAF-stimulated platelets. A 20 min preincubation with the PKC inhibitors completely abrogated the effect on $[Ca^{2+}]_i$ caused by PMA.

TxB_2 generation. With 10 μM-Ro 31-7549/001 and 3 μM-Ro 31-8220/002 (concentrations which inhibited PKC substrate phosphorylation by > 90%), the effect of inhibition of PKC on

Table 2. Effect of 10 μM-Ro 31-7549/001 and 3 μM-Ro 8220/002 on TxB_2 generation in 30 nM-PAF-stimulated platelets and the abrogation of PMA-induced inhibition of TxB_2 generation by the PKC inhibitors

Platelets were preincubated with the PKC inhibitors or vehicle 20 min before addition of the PKC activator PMA (30 nM) which was incubated for 2 min before stimulation with 30 nM-PAF. TxB_2 generation was measured at 3 min after PAF.

	TxB_2 (pmol/10^8 platelets)	
	Vehicle	+30 nM-PMA
Control	15.6±1.8	4.4±0.5**
10 μM-Ro 31-7549/001	65.1±10**	51.3±1.9**
3 μM-Ro 31-8220/002	50.9±9.9*	56.2±10*

TxB_2 generation was investigated in platelets stimulated with 30 nM-PAF (Table 2). Both PKC inhibitors caused a 3–5-fold increase in TxB_2 induced by 30 nM-PAF. Similarly to the results for $[Ca^{2+}]_i$ elevation, inhibition of TxB_2 generation by PMA was abrogated by the PKC inhibitors (Table 2). These findings indicate that the inhibition of $[Ca^{2+}]_i$ elevation and TxB_2 generation by PMA in PAF-stimulated platelets is mediated by PKC and can be reversed by preincubation with either PKC inhibitor.

5-HT release. The 5-HT release induced by 3 nM-PAF was dose-dependently inhibited when platelets were subjected to a 20 min preincubation with increasing concentrations of Ro 31-7549/001 (0.3, 1, 3, 10 μM) or Ro 31-8220/002 (0.1, 0.3, 1, 3 μM). The IC_{50} for Ro 31-7549/001 was 4.7 μM, and for Ro 31-8220/002 it was 0.55 μM (Fig. 3).

Aggregation. Aggregation of platelets induced by a submaximal concentration of PAF (1 nM) was dose-dependently inhibited with increasing concentrations of either PKC inhibitor, with almost total inhibition of platelet aggregation at 10 μM-Ro

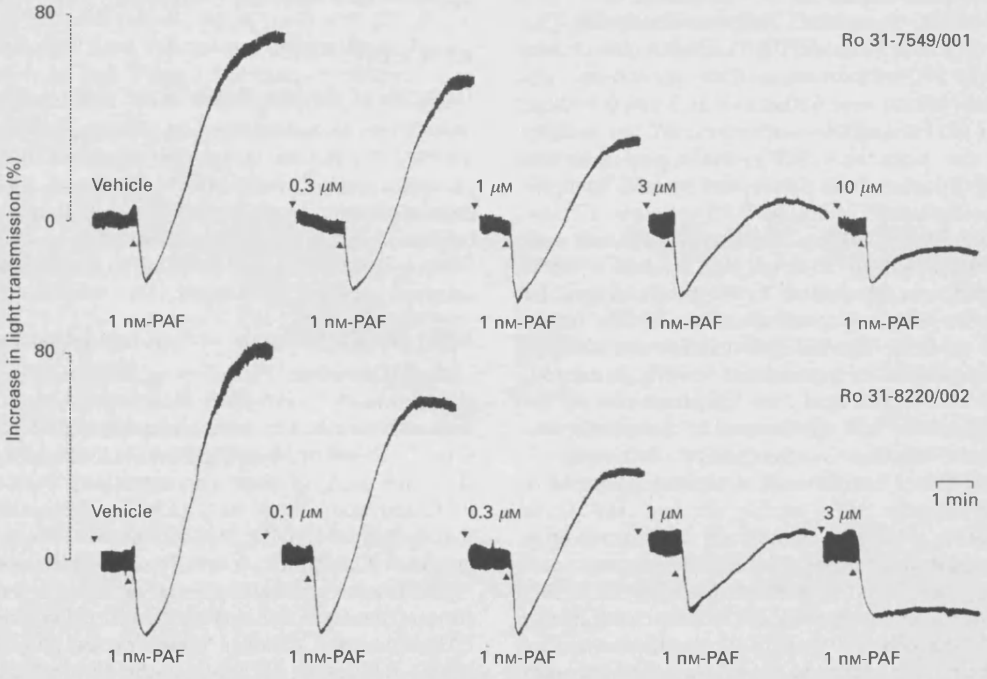

Fig. 4. Traces showing the effect of PKC inhibition on PAF-induced platelet shape change and aggregation

Platelets were preincubated for 20 min with a dose range of Ro 31-7549/001 (0.3–10 μM, top panel) or Ro 31-8220/002 (0.1–3 μM, lower panel). The second arrow in each trace indicates addition of PAF.

31-7549/001 or 3 μM-Ro 31-8220/002. The IC_{50} for Ro 31-7549/001 was 1.8 μM and for Ro 31-8220/002 it was 0.47 μM (Fig. 4). However, the PKC inhibitors did not affect 1 nM-PAF-induced shape change in platelets.

DISCUSSION

The use of the selective PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002 (Davis *et al.*, 1989) in this study has demonstrated that PKC has a bifurcating role in PAF-induced platelet activation. Earlier studies by both ourselves (Murphy *et al.*, 1990, 1991) and others (Conolly *et al.*, 1990; Watson & Lapetina, 1985) demonstrated that activating PKC with exogenous activators such as phorbol esters or membrane-permeant DAG, followed by receptor-operated platelet activation, leads to an inhibition of the two products of phospholipase C (PLC) activity, namely Ins(1,4,5)P_3 and DAG. This suggests that activated PKC is exerting a negative-feedback role which results in the inhibition of PLC either by inactivation of the receptor by phosphorylation, or at the level of the GTP-binding proteins coupling PLC to the receptor, or as a direct inactivation of the PLC enzyme.

In addition, other forms of platelet activation are inhibited as a consequence of exogenous PKC activation in agonist-stimulated platelets including $[Ca^{2+}]_i$ elevation (Poll & Westwick, 1986*a*; Valone & Johnson, 1987; Murphy & Westwick, 1989) and TxB_2 generation (Murphy & Westwick, 1989, 1990; Wheeler-Jones *et al.*, 1990). Inhibition of both these responses is possibly a direct result of inhibition of PLC and the production of the two signal molecules Ins(1,4,5)P_3 and DAG. Ins(1,4,5)P_3 causes mobilization of Ca^{2+} from intracellular stores (Brass & Joseph, 1985; Berridge & Irvine, 1984), and together with Ins(1,3,4,5)P_4 possibly regulates second-messenger-operated Ca^{2+} channels (Irvine, 1989, 1990). The inhibition of receptor-operated Ca^{2+} influx and Ca^{2+} mobilization would prevent the activation of Ca^{2+}-dependent phospholipase A_2 (PLA_2), which is probably responsible for most of the TxB_2 production (Mahaevappa & Holub, 1986). In addition, inhibition of PLC would remove the second source of arachidonic acid production and therefore TxB_2 generation, which is the action of DAG lipase on DAG (Bell *et al.*, 1979; Mahaevappa & Holub, 1986). However, recently there is increasing evidence to suggest that PLA_2 is also coupled to receptors by intermediate G-proteins, and it may be that PKC is exerting a negative feedback on PLA_2 via these G-proteins or indeed acting directly on PLA_2 (Silk *et al.*, 1990; Kajiyama *et al.*, 1989).

Nevertheless, if endogenously activated PKC is also exerting this negative-feedback effect, then inhibition of PKC would be expected to potentiate both $[Ca^{2+}]_i$ elevation and TxB_2 generation in receptor-activated platelets. In this study, two selective inhibitors of PKC employed at a concentration which caused greater than 90% inhibition of PKC substrate phosphorylation considerably potentiated the duration of $[Ca^{2+}]_i$ elevation, particularly at the higher concentrations of PAF; however, the maximal $[Ca^{2+}]_i$ elevation was not significantly altered. These results suggest that a potentiation of maximal $[Ca^{2+}]_i$ elevation with either PKC inhibitor is not seen in high-dose PAF-stimulated platelets, either because the fluorescent dye used in this study has become saturated, or because maximal elevation of $[Ca^{2+}]_i$ has been achieved and further elevation of Ins(1,4,5)P_3 cannot release any additional Ca^{2+}, but simply increases the duration of the signal molecule. Another possibility is that the Ins(1,4,5)P_3 5-phosphatase is normally activated by PKC, thus rapidly decreasing the cytosolic concentration of Ins(1,4,5)P_3. We have recently demonstrated that staurosporine, the non-selective PKC inhibitor, produces a significant enhancement of the Ca^{2+} signal (Murphy *et al.*, 1991), which was accompanied by a 2–3-fold increase in the products of PLC activation, namely Ins(1,4,5)P_3 and DAG.

The increase in duration of $[Ca^{2+}]_i$ elevation relates well to earlier findings that activation of PKC decreases the duration of $[Ca^{2+}]_i$ elevation either by increasing $[Ca^{2+}]_i$ efflux or decreasing $[Ca^{2+}]_i$ influx or mobilization (Poll & Westwick, 1986*b*; Pollock *et al.*, 1987), therefore removal of the effect of PKC activation with an inhibitor has produced the opposite effect. The more pronounced effect at the higher concentration of PAF may be due to inhibition of a greater negative feedback induced by a higher concentration of PAF; indeed, without this negative feedback at the higher concentration of PAF it is possible that Ca^{2+} may reach cytotoxic concentrations. As predicted, if endogenously activated PKC is exerting a negative feedback over PLC and/or PLA_2, TxB_2 generation was potentiated by inhibition of PKC. Moreover, *in vivo* the 3–5-fold increase in TxB_2 production, and thus thromboxane A_2 and prostaglandin endoperoxides, could have considerable effects, depending on the availability of prostacyclin synthase or vascular tissue responsive to the vasoconstrictor actions of thromboxane A_2 (Moncada & Higgs, 1986).

In contrast with the above results, this study has demonstrated that two selective inhibitors of PKC are potent inhibitors of dense-granule release and platelet aggregation, with similar IC_{50} values to those for the inhibition of PAF-induced phosphorylation of 40–47 kDa protein in intact platelets. These findings, together with the data demonstrating the specificity of these compounds, support the contention that inhibition of platelet dense-granule release and aggregation is a result of an inhibition of platelet PKC. In support of this, previous studies have shown that activation of PKC with a phorbol ester alone causes both dense-granule release and platelet aggregation (Zucker *et al.*, 1974; Castagna *et al.*, 1985), and it has been suggested that there is a direct link between phosphorylation of the major 40–47 kDa substrate of PKC and dense-granule release and aggregation (Rink *et al.*, 1983; Castagna *et al.*, 1985; Watson *et al.*, 1987).

Our results suggest that the relationship between PKC and $[Ca^{2+}]_i$ or TxB_2 generation is different from that between PKC and dense-granule release or platelet aggregation. In addition, the results indicate that the regulation of dense-granule release and aggregation by PKC is possibly independent of $[Ca^{2+}]_i$ or TxB_2 generation. Indeed, there appears to be a more direct relationship between PKC-induced protein phosphorylation and either dense-granule release or aggregation.

Comparing the effects of these selective PKC inhibitors on PAF-induced platelet activation with the effects of the non-selective PKC inhibitor staurosporine, many of the findings were similar. Both selective and non-selective inhibitors potentiated TxB_2 generation and the duration of the Ca^{2+} signal (Murphy *et al.*, 1991; Watson *et al.*, 1987), while inhibiting 5-HT release (Murphy & Westwick, 1989) and platelet aggregation (Oka *et al.*, 1987; Watson *et al.*, 1987; Schachtele *et al.*, 1988). However, whereas in platelets activated with sub-maximal concentrations of PAF staurosporine was found to inhibit platelet aggregation only partially (Watson & Hambleton, 1989), Ro 31-7549/001 and Ro 31-8220/002 caused total inhibition of aggregation while not affecting platelet shape change. It is therefore possible that staurosporine was inhibiting another protein kinase which was counteracting the effect of PKC inhibition on platelet aggregation. For example, increasing cyclic AMP levels activates protein kinase A, which inhibits platelet aggregation (Blache *et al.*, 1987); inhibition of protein kinase A by staurosporine would therefore remove this inhibitory effect and may therefore lead to only a partial possible inhibition of aggregation by staurosporine. The findings suggest therefore that sub-maximal-dose PAF-

induced platelet aggregation is PKC-dependent, whereas PAF-induced platelet shape change is PKC-independent.

In conclusion, the use of selective PKC inhibitors in PAF-stimulated rabbit platelets has demonstrated a bifurcating role for PKC. Ro 31-7549/001 and Ro 31-8220/002 were both selective inhibitors of platelet PKC and were potent inhibitors of PAF-induced dense-granule release and platelet aggregation, while producing a significant prolongation of the Ca^{2+} signal and a considerably enhanced TxB_2 generation.

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MODULATION OF RABBIT PLATELET THROMBOXANE B_2 GENERATION BY PROTEIN KINASE C

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We and others have shown that activators of protein kinase C (PKC) inhibit fluoride or agonist-stimulated thromboxane B_2 generation by human platelets (Poll et al., 1986,1987; Krishnamurthi et al., 1986). The aim of this study was to compare the effect of a PKC activator, TPA with a PKC inhibitor, staurosporine (Tamaoki et al.,1986) on rabbit platelet TxB_2 generation induced by PAF, ionomycin or arachidonic acid (AA). Cytosolic free calcium concentration ($[Ca^{++}]_i$), dense granule release and TxB_2 generation were measured as described previously (Poll et al., 1986) using prostacyclin washed rabbit platelets loaded with Fura 2. Results are the mean ± se mean of 3-6 experiments.

Incubation of platelets with 1, 3, 10 and 30 nM 12-0-tetradecanoyl phorbol 13-acetate (TPA) for two mins reduced submaximal PAF (3 nM) elevated $[Ca^{++}]_i$ from 455 ± 34.5 to 400.5 ± 4.2, 303 ± 26, 157 ± 11.5 and 21 ± 6 nM respectively and the corresponding TxB_2 generation (30 nM PAF) from 18.4 ± 1.8 to 19.77 ± 1.4, 6.7 ± 1.0, 2.4 ± 0.5 and 0.64 ± 0.1 pmol/10^8 platelets respectively. However TPA produced only a weak non-significant inhibition of PAF induced 5HT release. In contrast, TPA at 1, 3, 10, and 30 nM increased ionomycin (5µM)-induced TxB_2 generation from 161.2 ± 9.7 to 137.6 ± 31.7, 200.6 ± 9.3, 254 ± 29 and 259.6 ± 12 p mol/10^8 platelets respectively and 5HT release was potentiated from 59.6 ± 2.24 to 75.4 ± 5.9, 84.8 ± 17.1, 95.85 ± 4.1 and 99.6 ± 0.4% respectively. The AA (10 µM)-induced elevation of $[Ca^{++}]_i$ was reduced from 192 ± 17 to 143 ± 19, 29 ± 13.5 with no detectable $[Ca^{++}]_i$ elevation when TPA values reached 10 nM and above. With all concentrations of TPA examined AA-induced TxB_2 generation was not modified ($p>0.05$).

Incubation of platelets with 30, 100, 300 and 1000 nM staurosporine for two minutes did not modify PAF (3nM) elevated $[Ca^{++}]_i$ (N=4) nor was there any significant potentiation of TxB_2 generation with 30 nM PAF except with 1 µM staurosporine. However, 5HT release with 3 nM PAF was inhibited from 58.4 ± 2.5 to 52.7 ± 6, 46.1 ± 6.6, 43 ± 3.7 and 20.1 ± 8.1 % respectively (N=4). As with TPA, staurosporine did not modify $[Ca^{++}]_i$ elevated by 5 µM ionomycin (N=3) however in direct contrast to the results obtained with TPA, TxB_2 generation with 5 µM ionomycin was inhibited from 151.4 ± 13.4 to 142.3 ± 30, 79.5 ± 9.7, 66.8 ± 7.4 and 34.8 ± 9.8 pmol/10^8 platelets respectively, and 5HT release was reduced from 54.4 ± 3.5 to 47.2 ± 4.7, 42.2 ± 0.4, 8.8 ± 6.5 and 6.15 ± 1.6 %. Staurosporine (30-1000 nM) as with TPA did not modify the amount of TxB_2 generated in response to submaximal AA (30 µM) (N=3).

Therefore these results suggest that receptor stimulated elevation of $[Ca^{++}]_i$ and TxB_2 generation are not controlled by endogenous PKC activators, as demonstrated with staurosporine. Although if the $[Ca^{++}]_i$ is not limiting, as with ionomycin, TxB_2 generation can be markedly enhanced or inhibited with PKC activators or inhibitors respectively. The site of modulation by PKC is probably not the cyclo-oxygenase or thromboxane synthetase enzymes as AA-induced TxB_2 generation is not modified.

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CHARACTERISATION OF PLATELET-ACTIVATING FACTOR-INDUCED DIACYLGLYCEROL GENERATION IN RABBIT PLATELETS

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We have previously demonstrated that ionomycin-induced 5-HT release and thromboxane B_2 (TxB_2) generation are readily modulated by activation or inhibition of protein kinase C (PKC). However, sub-optimal platelet-activating factor (PAF)-induced elevation of cytosolic calcium concentration ($[Ca^{++}]_i$), 5-HT release and TxB_2 generation are not similarly modified (Murphy & Westwick, 1989). To analyse this paradox, we have determined PAF-induced formation of diacylglycerol (DAG), the endogenous activator of PKC. DAG was measured using the Amersham kit; $[Ca^{++}]_i$ and TxB_2 generation were measured as previously described (Murphy & Westwick, 1989; Poll *et al.*, 1986) in rabbit platelets.

Addition of 300nM PAF produced a rapid elevation of DAG concentration, which peaked at 10s; the values at 5, 10, 15, 20, 30 and 60s were 86.8±15, 280±52, 65±11, 87±31, 66±18 and 69±10 pmoles/10^9 platelets, above the basal value of 117±3 (n=3-9). This high concentration of PAF produced a peak elevation of $[Ca^{++}]_i$ of 1023 ± 72 nM, which occurred at 7.3±0.6s (n=13) and 62.7±12.9% of total TxB_2 was produced within 10s. At 10s after the addition of 0.3, 3, 30 and 300 nM PAF, the generation of 18±6, 8±6, 80±19 and 280±52 pmol DAG/10^9 platelets (n=3-9) above basal values was induced. Thus sub-optimal concentrations of PAF (0.3-3nM) do not increase DAG above basal values. Therefore, it is unlikely that PKC can exert a negative feedback on low-dose PAF-activated systems. For example, pre-treatment of platelets for 2 min with 1μM staurosporine (an inhibitor of PKC, Tamaoki *et al.*,1986), caused an approximately 3-fold enhancement of 300 nM PAF-induced DAG level, from 413±8.5 to 1147±227, but had no significant effect on 3nM PAF-induced DAG level, from 112.5±7 to 146±35 pmol/10^9 platelets. These results are paralleled by the staurosporine (1μM)-induced tripling of duration of 300nM (but not 3nM) PAF-elevated $[Ca^{++}]_i$ from $t_{½}$ 34±4 to 105±4 s. Similarly, the 300nM PAF-induced TxB_2 generation is doubled by pretreatment with 1μM staurosporine from 170±19 to 307±17 pmol TxB_2/10^8 platelets.

Pretreatment with exogenous PKC activator 12-0-tetradecanoyl phorbol 13-acetate (100nM) reduced 3nM and 300nM PAF-induced DAG formation from 112±5 to 45±32 and from 413±8.5 to 140±37.4 pmol/10^9 platelets respectively.

Thus, with high concentrations of PAF, the diacylglyerol levels, duration of elevation of $[Ca^{++}]_i$ and generation of TxB_2 are significantly enhanced by inhibition of PKC.

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CHARACTERISATION OF PLATELET ACTIVATING FACTOR INDUCED INOSITOL (1,4,5)-TRISPHOSPHATE PRODUCTION IN PLATELETS: MODULATION BY PROTEIN KINASE C

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In a previous study we found that ionomycin-stimulated platelet activation was readily modified by protein kinase C (PKC) activation or inhibition, however this was not true for sub-maximal PAF stimulation (Murphy & Westwick, 1989a). Further investigations which examined PAF-induced formation of diacylglycerol (DAG) (Murphy & Westwick, 1989b), the endogenous activator of PKC, suggested that there was a disparity between DAG production and functional response. We have investigated these findings by examining the effect of staurosporine (the potent although non-specific PKC inhibitor - Tamaoki et al, 1986) on PAF induced inositol-(1,4,5)-trisphosphate (IP_3) production in rabbit platelets via use of the Amersham binding assay, protein phosphorylation in ^{32}P labelled platelets and cytosolic calcium ($[Ca^{++}]_i$) elevation in Fura-2 loaded platelets.

Addition of 300 nM PAF produced a rapid elevation in IP_3 concentration which peaked at 2s with values of 45.8 2.2, 21.7 6.9, 6.9 4, 1.3 1.7, 1.1 1.6, 2.5 2.1 and 9.0 3.1 pmols $IP_3/10^9$ platelets at 2,5,10,15,20,30 and 60s respectively, above a basal level of 11.8 0.6 pmols/10^9 platelets. A peak elevation of $[Ca^{++}]_i$ of 1023 72 nM occurred at 7.3s after stimulation with 300 nM PAF. The peak IP_3 production therefore precedes the peak elevation of $[Ca^{++}]_i$ induced by 300 nM PAF.

Using concentrations of PAF (0.3,3,30,300 nM) which increased maximal $[Ca^{++}]_i$ to 199 45, 435 45, 628 34 and 1023 72 nM respectively above a basal concentration of 109 9 nM we measured IP_3 production. At 5 seconds, 0.3,3,30 and 300 nM PAF were found to generate 8.8 1.0, 12.7 1.3, 23.6 4.8 and 30.2 1.9 pmols $IP_3/10^9$ platelets respectively, compared with the basal value of 11.8 0.6 pmols/10^9 platelets. Thus sub-optimal concentrations of PAF do not appear to increase IP_3 above basal concentrations, these results are in agreement with earlier findings that sub-optimal concentrations of PAF (0.3,3 nM) did not increase DAG above resting levels. Indeed, in support of this we have found that there is a good correlation between DAG production and the ability of 0.3,3,30, and 300 nM PAF to phosphorylate the 40-47 kDa protein substrate of PKC.

The effect of PKC inhibition on IP_3 elevation was investigated using staurosporine. Dose response studies demonstrated 1 μM staurosporine to almost completely inhibit 300 nM PAF-induced PKC substrate phosphorylation whilst having no effect on resting IP_3 levels. At 5 seconds post-PAF a 2 minute pre-incubation with 1 μM staurosporine induced a 2 fold potentiation in 300 nM stimulated IP_3 levels from 30.2 1.9 to 55.15 3.8 pmols/10^9 platelets, whilst having no effect on 3 nM PAF stimulated IP_3 with values of 12.7 1.3 and 9.5 0.5 pmols $IP_3/10^9$ platelets for control and staurosporine pre-treated respectively. Moreover, we investigated the effect of staurosporine (1 μM) on maximal $[Ca^{++}]_i$ elevation and on the $T_{1/2}$ (the time for elevated $[Ca^{++}]_i$ levels to decline to 1/2 maximal) in PAF-stimulated platelets. Staurosporine had no effect on maximal height of $[Ca^{++}]_i$ induced by either 3 or 300 nM PAF, however in accord with the effect of staurosporine on IP_3 production, it induced a 200% increase in the $T_{1/2}$ of 300 nM PAF elevated $[Ca^{++}]_i$ whilst inducing only a 30% increase in the $T_{1/2}$ of 3 nM PAF elevated $[Ca^{++}]_i$.

In conclusion, there is a good correlation between the effect of PKC inhibition on IP_3 production and DAG production induced by different concentrations of PAF. Thus platelet activation by lower concentrations of PAF may not involve phosphoinositide hydrolysis or activation of PKC.

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EFFECT OF PROTEIN KINASE C MODULATION ON TXB_2 GENERATION IN PAF AND IONOMYCIN STIMULATED RABBIT PLATELETS. Murphy, C.T. and Westwick, J. Department of Pharmacology, Royal College of Surgeons, London, WC2A 3PN, UK.

This study aimed to compare the effect of a PKC activator, 12-0-tetradecanyl-phorbol 13-acetate (TPA) with a PKC inhibitor, staurosporine (SS) on platelet cytosolic calcium ($[Ca^{++}]_i$) elevation and TxB_2 generation induced by PAF or ionomycin.

Pre-incubation of platelets with 1-30 nM TPA reduced sub-maximal PAF (3 nM) elevated $[Ca^{++}]_i$ by 95% and correspondingly TxB_2 generation (30 nM PAF) was inhibited by almost 100%. In contrast however, whereas TPA had no effect on ionomycin (5 μM) induced $[Ca^{++}]_i$ elevation, it potentiated TxB_2 generation upto 1.6 fold. A concentration range of SS (30-1000 nM) which dose dependently inhibited PKC activation (measured by inhibition of PAF induced phosphorylation of the 47 KDa protein substrate of PKC) did not modify maximal $[Ca^{++}]_i$ elevation induced by 3 nM PAF however, it dose dependently reversed the inhibitory effect of 30 nM TPA on 3 nM PAF induced $[Ca^{++}]_i$ elevation. TxB_2 generation was significantly potentiated at the highest concentration of SS used (1 μM). As with TPA, SS did not modify ionomycin induced $[Ca^{++}]_i$ elevation however in direct contrast to the results obtained with TPA, TxB_2 generation was inhibited bt 78%. Neither TPA nor SS modified arachidonic acid-induced TxB_2 generation indicating that neither cyclo-oxygenase or TxB_2 synthetase is likely to be the site of PKC modulation. Replacing $[Ca^{++}]_e$ with EGTA and thereby inhibiting PAF-induced $[Ca^{++}]_i$ elevation significantly inhibited TxB_2 generation suggesting that the inhibition of $[Ca^{++}]_i$ elevation by TPA may correlate with the subsequent inhibition in TxB_2 generation. However, when $[Ca^{++}]_i$ is unaffected by PKC modulation as in ionomycin stimulated platelets TxB_2 generation can be directly modulated by PKC.

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CHARACTERISATION OF PAF-INDUCED DIACYLGLYCEROL AND INOSITOL (1,4,5)-TRISPHOSPHATE PRODUCTION IN RABBIT PLATELETS. Murphy, C.T. and Westwick, J. Department of Pharmacology, Royal College of Surgeons, London WC2A 3PN, UK.

We have explored the role of endogenous protein kinase (PKC) activators in regulating the levels of diacylglycerol (DAG), inositol (1,4,5)-trisphosphate (IP_3) and cytosolic calcium ($[Ca^{++}]_i$) in PAF stimulated rabbit platelets.

300 nM PAF produced a rapid and transient elevation in DAG , IP_3 and $[Ca^{++}]_i$ levels. DAG peaked at 10s with a 2.4 fold increase, IP_3 peaked at 2s with a 3.8 fold increase and $[Ca^{++}]_i$ peaked at 7.3 s with a 10 fold increase over basal levels. Sub-maximal concentrations of PAF (0.3, 3 nM) elevated $[Ca^{++}]_i$ but did not elevate either DAG or IP_3 above basal levels. However, 30 and 300 nM PAF induced a dose dependent increase in DAG, IP_3 and $[Ca^{++}]_i$. PKC activity measured by PAF-induced phosphorylation of the 47 KDa protein substrate of PKC supported these findings. Inhibition of PKC with 1 μM staurosporine (SS) potentiated DAG levels 3.6 fold and IP_3 levels 2 fold in 300 nM PAF stimulated platelets, but had no effect in platelets stimulated with 3 nM PAF. 1 μM SS also induced a 200% increase in the time for elevated $[Ca^{++}]_i$ to decay to 1/2 maximal ($T_{1/2}$) whilst only inducing a 30% increase in the $T_{1/2}$ of 3 nM PAF elevated $[Ca^{++}]_i$. Conversely, pretreatment with exogenous PKC activator 12-0-tetradecanylphorbol 13-acetate (100 nM) inhibited 3 and 300 nM PAF-induced DAG levels by 60% and 66% respectively.

In conclusion, it is unlikely that PKC can exert a negative feedback in low dose PAF-activated systems, as there is no DAG elevation, and neither IP_3 nor DAG levels are elevated by PKC inhibition.

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Modulation of the activation of platelets by genistein, an inhibitor of tyrosine kinase(s)

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We have explored the role of tyrosine kinases in the signal transduction of PAF-activated rabbit platelets. Rabbit platelets were prepared, signal molecules quantitated and dense granule release determined as described previously (Murphy et al, 1991). Tyrosine phosphorylation of rabbit platelet proteins was determined by Western blotting of platelet lysates with PY20, a monoclonal anti-phosphotyrosine antibody (Glenney et al, 1988). Resting platelets exhibited at least four tyrosine phosphorylated (TP)-proteins of 52-62 kDa. Stimulation with 300 nM PAF induced a rapid increase in TP-proteins visible within 5 secs of both low (35-45 kDa) and high (66-90 kDa) molecular weight. Pretreatment of platelets with genistein (10-300 μM) for 20 min produced a dose related inhibition of tyrosine phosphorylation of a number of the 66-150 kDa and 35-45 kDa proteins, although was much less effective against the proteins of 52-62 kDa. Genistein (Akiyama et al, 1987) was an effective inhibitor of PAF-induced calcium elevation ($[Ca^{++}]_i$), inositol 1,4,5-trisphosphate (IP_3) formation, thromboxane (Tx) B_2 generation and the release of dense granules (^{14}C-5HT) see table.

Treatment	$[Ca^{++}]_i$ (nM)	IP_3(pmol/10^9pl)	TxB_2(pmol/10^8pl)	^{14}C-5HT (%)
Unstimulated	107 ± 12	7.5 ± 1.5	<0.1	<0.1
Vehicle + PAF	490 ± 22	34 ± 5.5	44 ± 3.2	58 ± 1.5
10 μM Genistein + PAF	434 ± 17.5	-	25.5 ± 0.31	46 ± 0.7
30 μM Genistein + PAF	421 ± 15	22 ± 2	3.4 ± 1.6	39 ± 2.8
100 μM Genistein + PAF	341 ± 24	15 ± 0.8	<0.1	13.8 ± 2.3
300 μM Genistein + PAF	135 ± 13	10.2 ± 2.7	<0.1	3 ± 0.5

We have demonstrated that PAF induces a rapid tyrosine phosphorylation of approximately 17 proteins in three molecular weight ranges. This probably involves a number of tyrosine kinases as genistein is an effective inhibitor of only a limited number of proteins. However, these TP proteins appear to be crucial in the very early signal transduction events of PAF activated platelets as genistein is an effective inhibitor of these events.

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